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OBSERVATIONS ON THE INFLUENCE OF PLANT ILLUMINATION ON THE FUNGAL FLORA OF ROOTS[1]

E. A. PETERSON

Abstract

Greenhouse and plant growth room experiments showed that shading of plants had no appreciable effect on the vegetatively active fungi colonizing the primary roots of wheat and soybean seedlings growing in fertile, disease-free soil. Although marked differences in plant development were obvious, the general pattern of root colonization was essentially the same for the two levels of illumination used. The results suggest that the saprophytic fungi which normally colonize the roots of healthy plants may also colonize roots of abnormal plants providing that the soil is relatively free from plant pathogenic forms.

Species of *Phoma* accounted for a much higher proportion of the isolates from wheat roots than from soybean roots, whereas the majority of cultures from soybeans proved to be species of *Fusarium*.

Introduction

The fungal flora associated with plant roots is subject, either directly or indirectly, to the influence of a number of factors. The development of distinct soil mycofloras under different crops (6) and the occurrence of qualitative differences in the fungal populations of the rhizosphere of different varieties of individual plant species (1, 11) attests to the effect of plant type. The mycoflora of the root zone changes as the plant grows (5) with certain fungi assuming predominance, especially on the root surface or rhizoplane (7, 9). Such effects may be attributed largely to inherent differences in chemical composition, nutrition, and physiology among plant species and varieties as reflected primarily in the nature of their root excretions.

Apart from the plant itself, soil type, reaction, and treatment have been shown to affect the fungal flora of roots (5, 7, 10). Light intensity also appears to have important indirect effects on certain fungi in the soil. For example, low light intensity has been shown to increase the susceptibility of tomato plants to *Fusarium* wilt (2). The incidence of damping-off of tree seedlings has been reported to increase as a result of shading the seedbeds (12). Harley

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Contribution No. 504, from the Microbiology Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Canada.

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and Waid (4) observed that shading of beech seedlings caused distinct changes in the fungal population of the roots, notably an increase in the numbers of semipathogens.

During the present study an attempt was made to obtain further information on the influence of plant illumination on the normal fungal flora of roots in the absence of known disease factors. Results of fungal analyses for wheat and soybeans are presented in this report.

Methods

Greenhouse

For preliminary tests, wheat and soybeans were sown in beds of a fertile field soil in the greenhouse. After 2 weeks of growth under the normal light of the greenhouse, half of the seedlings of each crop were shaded with a cheesecloth cage, the light being reduced from approximately 1000 to 300 foot-candles. Soil moisture was maintained by daily sprinkling. Root samples for analysis were taken from four locations in each treatment 3 weeks after reduction of light.

Growth Room

Wheat and soybeans were sown in 6-in. pots filled with fertile field soil (as above) which had been thoroughly mixed and sieved. Immediately after seeding, the pots were placed in an air-conditioned growth room which provided uniform temperature and humidity. One-half of the pots of each crop were subjected to a uniform light intensity of 1200 foot-candles, the other half to 300 foot-candles for 14 hours each day. Pots were watered daily by sprinkling. Throughout the experiment the temperature at soil level was maintained at 65–68° F and the relative humidity of the air at 60–70%. Samples for analysis were taken at 4 and at 7 weeks after seeding.

Fungal Determinations

Approximately 60 seedlings were removed from the soil from each treatment. One 4-cm length of primary root was cut from each plant immediately below the crown. After removal of lateral rootlets, the root lengths were serially washed in 20 changes of sterile water to remove dormant fungal spores and other easily detached material. Two 0.5-cm segments cut from opposite ends of each root length were plated on peptone dextrose agar containing rose bengal and chlortetracycline in order to isolate fungi which remain associated with the roots mainly in a vegetative state. All plates were incubated at 20° C. Other details of procedure have been described previously (7).

Results

In the preliminary greenhouse tests conducted in the spring of 1959, visible effects of shading were evident in both wheat and soybean seedlings when sampled 3 weeks after the reduction of light. Shaded plants showed abnormal stem elongation with a tendency to lodge, slight leaf chlorosis, and poor root development in contrast to the deep green, well-developed seedlings growing at the higher light intensity.

TABLE I

Percentage incidence of fungi associated with roots of plants grown in the greenhouse under two levels of illumination

Predominant genera	Wheat		Soybeans	
	1000*	300	1000*	300
Rhizopus	—	1.3	9.4	6.4
Pythium	—	—	2.4	8.8
Phoma	44.3	23.7	1.6	4.0
Penicillium	—	3.9	1.6	0.8
Pullularia	4.1	9.2	—	1.6
Periconia	10.3	—	—	—
Helminthosporium	3.1	1.3	0.8	—
Fusarium	13.5	47.3	74.0	71.2
Cylindrocarpon	1.0	—	—	3.2
Total no. of isolates	97	76	127	125

*Approximate light intensity (foot-candles).

The results of fungal determinations summarized in Table I show some changes in predominance of certain fungi associated with the roots of wheat seedlings, which may be a reflection of the visible differences in plant development resulting from shading. The relative incidence of *Fusarium* spp. was found to be much higher on the roots of shaded wheat seedlings than on those of the seedlings grown under normal light. It was of special interest to note that the well-known root pathogen *Fusarium culmorum* accounted for 28% of the fungi obtained from the roots of shaded plants as compared with 3% from the roots of plants under the higher light intensity. It is difficult to explain the lower incidence of fungi such as *Phoma* and *Periconia* in terms of shading. However, it should be pointed out that rapidly growing fungi such as *F. culmorum* often overgrow and obscure the more slowly growing forms on agar media. Therefore, due to limitations of the technique employed, the proportions of slowly growing fungi may be underestimated on root segments which are also occupied by rapidly growing types.

In spite of the marked effect of shading on soybean seedlings the pattern of fungal colonization of roots was similar for both normal and shaded plants. Only a slight increase in the incidence of *Pythium* spp. on the roots of shaded seedlings was noted.

In order to avoid the wide fluctuations of light, temperature, and humidity experienced in the greenhouse, a pot experiment was conducted in an air-conditioned plant growth room in the autumn of 1959. Light was reduced over the shaded series at the time of seeding to permit maximum opportunity for the expression of any influence on fungi which colonize the roots at the beginning of their growth.

Figure 1 shows the visible effects of shading on the development of wheat and soybean seedlings 6 weeks after seeding. As noted earlier in greenhouse tests, shaded plants were spindly and required support to prevent lodging. Leaves were chlorotic and root systems were poorly developed. Stems and roots lacked normal fiber, being readily broken off. Nodules were not observed on the roots of shaded soybean seedlings, whereas they were numerous and well

TABLE II

Percentage incidence of fungi associated with roots of plants grown in a growth room under two levels of illumination

Predominant genera	Wheat		Soybeans	
	1200*	300	1200*	300
	4 weeks after seeding			
Rhizopus	7.4	2.4	14.1	16.9
Pythium	—	—	—	4.6
Phoma	20.4	29.3	7.0	8.5
Penicillium	2.8	6.5	0.8	1.5
Pullularia	9.3	4.1	—	2.3
Periconia	13.0	12.2	2.3	—
Helminthosporium	11.1	12.2	—	1.5
Fusarium	13.9	13.0	58.6	49.2
Cylindrocarpon	2.8	9.8	9.4	5.4
Total no. of isolates	108	123	128	130
	7 weeks after seeding			
Rhizopus	5.2	2.7	5.6	7.0
Pythium	—	4.1	—	—
Phoma	46.1	45.2	7.0	5.6
Penicillium	—	2.7	—	—
Pullularia	4.5	1.4	1.4	—
Periconia	13.0	2.7	1.4	—
Helminthosporium	3.2	6.8	—	—
Fusarium	8.3	16.5	76.0	85.2
Cylindrocarpon	2.6	—	6.3	2.1
Total no. of isolates	154	73	142	142

*Light intensity (foot-candles).

developed on the roots of the plants grown at the higher light intensity. Root systems were found to be generally clean on plants grown under both high and low light intensity.

Root samples from wheat and soybeans grown under two levels of illumination were taken 4 and 7 weeks after seeding. A summary of fungal determinations is given in Table II. It may be noted that the pattern of fungal colonization of roots is very similar for both the low- and the high-light series. The results obtained for soybeans are in close agreement with corresponding data from earlier greenhouse experiments. However, contrary to previous findings, the relatively high incidence of *Fusarium* spp., particularly *F. culmorum*, on the roots of shaded wheat seedlings was not observed during growth room studies. Although slight differences (of doubtful significance) in the proportions of fusaria were recorded for wheat root samples taken 7 weeks after seeding, *F. culmorum* was not represented among isolates from either the low- or high-light series. The incidence of other potential root parasites such as *Pythium*, *Helminthosporium*, and *Cylindrocarpon* did not appear to be altered appreciably by shading.

Apart from light as a factor influencing the fungal flora of plant roots, the

PLATE I

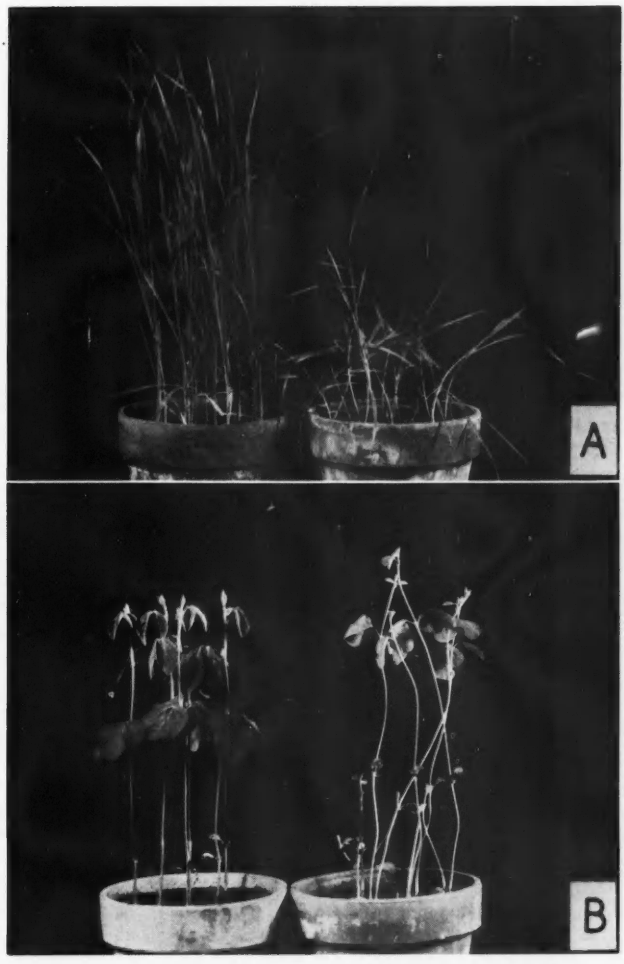

FIG. 1. Effect of light on plant growth: (A) wheat, (B) soybeans (left, 1200 foot-candles; right, 300 foot-candles).

effect of plant type is indicated by distinct differences in the incidence of *Phoma* and *Fusarium* spp. noted for wheat and soybeans. In both greenhouse and growth room tests, species of *Phoma* accounted for much higher proportions of the isolates obtained from wheat roots than from soybean roots. In contrast, the majority of cultures from soybean roots were found to be species of *Fusarium*, *F. oxysporum* being the dominant species.

Discussion

Results of greenhouse and plant growth room experiments showed that shading of plants had no appreciable influence on the vegetatively active fungi associated with the primary roots of wheat and soybean seedlings even though marked effects on plant development were evident. With the exception of the fungus *F. culmorum*, which accounted for a relatively high proportion of the isolates obtained from the roots of wheat seedlings under reduced light in the greenhouse, a general increase in the incidence of potential pathogens previously reported to result from shading (4, 12) was not observed. Instead, the pattern of root colonization was found to be similar at both levels of illumination tested.

It may be possible to explain the observed results, at least in part, in terms of "inoculum potential", defined by Garrett (3) as "the energy of growth of a fungus (or other microorganism) available for colonization of a substrate at the surface of the substrate to be colonized". Since the soil employed for these studies was a fertile field soil capable of supporting normal plant growth, it seems likely that the "inoculum potential" of semipathogens therein was below the level necessary for successful colonization of roots. It has been established (3) that the susceptibility of wheat seedlings to attack by species of *Fusarium* causing seedling blights increases with a rise in temperature. This may account for the failure to isolate *F. culmorum* from wheat roots during growth room tests in proportions comparable to those obtained under greenhouse conditions since the average temperature in the greenhouse was approximately 15° F higher than that of the growth room and fluctuated over a much greater range (55–95° F).

The qualitative changes in the nature of root excretions which may result from the shading of plants (8) do not necessarily imply major changes in the fungal flora of the roots. Many of the fungi which predominate on the roots of healthy plants growing in fertile soil are capable of utilizing a wide range of substrates for their nutrition. Therefore, with a relative absence of potential plant pathogens, the same saprophytic fungi which colonize the roots of healthy plants growing under conditions of normal light intensity might be expected also to colonize roots of plants growing under lower illumination even though the quality of the root excretions may have been altered. Indeed, such a view appears to be strongly supported by the results of the present studies.

Acknowledgment

The author gratefully acknowledges the effective technical assistance of Mr. Henry Malinowski.

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STUDIES OF AEROBIC NON-SYMBIOTIC NITROGEN-FIXING BACTERIA[1]

E. A. Paul and J. D. Newton

Abstract

The occurrence of aerobic, non-symbiotic, nitrogen-fixing bacteria was determined in samples of soil collected in the various soil zones of Alberta and Saskatchewan. Mannitol and sodium benzoate dust-plates, and mannitol solution cultures with subsequent inoculation onto mannitol agar demonstrated that *Azotobacter* were not widespread in the Canadian prairie province soils. These procedures also led to the isolation of smaller, aerobic, non-symbiotic, nitrogen-fixing organisms from all the Alberta and Saskatchewan soils studied. These smaller, nitrogen-fixing bacteria which developed as 1- to 3-mm circular, convex, unpigmented colonies on mannitol and glucose agar were classified as *Pseudomonas*. Flagellation of the 0.75 to 1 μ by 1.5- to 2-μ Gram-negative, coccoid rods was polar. Starch was hydrolyzed; gelatin was not liquified. Indol, acid, and gas were not produced; litmus milk was not reduced, but hydrogen sulphide was formed. The pseudomonads, capable of initiating growth at a pH of 4.9, could also grow at 8° C, whereas the *Azotobacter chroococcum* required higher temperatures and reactions above pH 6.2. *Azotobacter chroococcum* fixed up to 12 mg nitrogen per gram of carbohydrate. The smaller bacteria, classified as *Pseudomonas azotogensis*, fixed from 0.1 to 3.9 mg N per gram of mannitol.

Introduction

The agricultural soils of the Canadian prairies, developed primarily under grassland condition, have relatively high contents of organic matter and nitrogen. Because of the scarcity of legumes in the native prairie vegetation, the role of symbiotic nitrogen fixation in the accumulation of this nitrogenous organic matter is of doubtful significance, especially in the semiarid regions (9). The dry-land agriculture practised in this area does not include extensive nitrogen additions by either fertilization or legumes; maintenance of the nitrogen content therefore appears to depend largely on non-symbiotic nitrogen fixation.

The fact that agricultural soils of this region contain organisms capable of fixing atmospheric nitrogen has been previously demonstrated (8, 9, 10). Soils of the brown, black, and grey-wooded soil zones were found to fix significant quantities of nitrogen when incubated with plant residues.

Azotobacter were isolated from the irrigated soils of the brown soil zone (3). Their occurrence under conditions of dry-land cultivation was, however, found to be spasmodic. The black and grey-wooded soils, which Newton found to fix atmospheric nitrogen, did not contain *Azotobacter* (3, 9). Therefore, although it was known that these soils contained non-symbiotic nitrogen-fixing organisms, their characteristics had not been studied.

Methods

Organisms capable of developing on media without added nitrogen were

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Contribution from the Departments of Soil Science, University of Saskatchewan, Saskatoon, Saskatchewan, and University of Alberta, Edmonton, Alberta.

isolated from 44 cropped or virgin sites in Alberta and Saskatchewan. The soils studied ranged from the irrigated and non-irrigated semiarid soils of the brown soil zone to the subhumid grey-wooded zone.

The isolation media consisted of mannitol and sodium benzoate agar without added nitrogen (K_2HPO_4, 0.5 g; $MgSO_4.7H_2O$, 0.2 g; NaCl, 0.2 g; $CaSO_4.2H_2O$, 0.1 g; $FeSO_4.7H_2O$, 0.01 g; $Na_2MoO_4.7H_2O$, 0.05 g; agar 15 g; distilled water 1000 ml). Two per cent mannitol or 0.1% sodium benzoate served as the energy source. Approximately one-tenth gram of pulverized soil was shaken from a spatula onto the agar and incubated. A solution of mannitol with the necessary mineral salts was also inoculated with soil and subsequently subcultured onto mannitol and sodium benzoate agar.

The macroscopic growth characteristics and the microscopic morphological features were determined for organisms capable of fixing significant quantities of nitrogen as determined by a micro-Kjeldahl procedure. Unless otherwise specified, the techniques were those suggested by the Manual of Microbiological Methods (15).

Microscopic examination and growth on sugar-free peptone media were used for purity controls of the cultures. Unless otherwise specified, the incubation period was 7 days at 28° C.

Determination of Nitrogen Fixation

The nitrogen-fixing ability of the organisms was determined in a solution buffered at pH 7.2 by 1.8 g K_2HPO_4 and 0.7 g KH_2PO_4 per liter of 2% mannitol solution plus mineral salts. Two milliliters of a washed resting suspension of cells, adjusted to a concentration which gave a reading of 15% transmittance at 585 mμ on a "Spectronic 20" colorimeter, were added to 5 ml of mannitol solution. The controls immediately received 1.5 ml of concentrated H_2SO_4. The cultures were incubated at 25° C for 48 hours in a constant temperature room equipped with a reciprocating shaker.

The determination of total nitrogen employed the principles of a method developed by Polly (11). The cultures after incubation were digested with 1.5 ml concentrated H_2SO_4, 0.05 ml of 10% $HgSO_4$, and 0.5 g K_2SO_4. After 30 minutes' digestion, the solution was cooled and five drops of 30% hydrogen peroxide were added. Then digestion was continued for another 10 minutes. The flasks were allowed to cool, the mercury precipitated with sodium sulphide and aliquots of the supernatant diluted for Nesslerization.

Results

Azotobacter in Alberta Soils

Dusting mannitol and sodium benzoate agar plates with soil demonstrated, on incubation, that *Azotobacter* were present in the three irrigated soils collected from the brown soil zone, and in one adjacent cultivated non-irrigated soil. The other dry-land cultivated and the virgin soil sites were devoid of these nitrogen-fixing organisms.

Inoculation of mannitol solution with subsequent transfer onto mannitol and sodium benzoate agar verified that *Azotobacter* were absent from most of the Alberta soils tested. Characterization of the isolates obtained from the various soils showed that the *Azotobacter* produced large, mucous, coalescent

TABLE I

Atmospheric nitrogen fixed by *Azotobacter chroococcum* during 48 hours' incubation in 5 ml of 2% mannitol solution

Location of soils from which organisms were isolated	Nitrogen content		Nitrogen fixed	
	Initial	After incubation	μg/ml	mg/g of mannitol
Taber	0.18 (0.13–0.25)*	1.24 (1.19–1.35)*	212	10.6
Vauxhall	0.17 (0.17–0.18)	1.45 (1.19–1.58)	256	12.8
Brooks	0.19 (0.17–0.18)	0.35 (0.27–0.41)	35	1.65

*Range of four determinations.

colonies having a greyish-brown water-insoluble pigment. On sodium benzoate agar, 3- to 4-mm, flat, circular colonies with a black, water-soluble pigment were formed. The growth on mannitol agar slants was brown filiform showing evidence of slime production. On sodium benzoate slants filiform to beaded growth produced a black, soluble pigment.

Growth of the 2- to 3-μ, yeastlike *Azotobacter* cells was very limited in nutrient broth, in peptone water, and on nutrient agar. Potassium nitrate slightly inhibited growth on mannitol agar. Starch was utilized. Gelatin was not liquified, nor was indol produced from tryptophan. Hydrogen sulphide was not produced nor was litmus milk reduced after 14 days.

The *Azotobacter* were capable of initiating growth between pH values of 6.2 and 7.9 with an optimum growth rate at pH 7.0. The organisms developed more profusely at 28° C than at either 25° C or 37° C. Growth did not occur at 8° C. The thermal death temperature for a 10-minute exposure was 55° C.

Table I shows that approximately 12 mg nitrogen was fixed per gram of mannitol when a heavy, washed resting suspension of cells was inoculated into the energy source and incubated for 48 hours. One of the cultures tested, however, fixed only 1.65 mg of nitrogen under these conditions.

Other Nitrogen-fixing Organisms

Every one of the 72 individual samples of soil from the brown, dark brown, black, and grey-wooded soils of Alberta contained organisms, other than *Azotobacter*, capable of developing on media with no nitrogen added. The colonial characteristics, microscopic appearance and biochemical properties of the organisms isolated from the 0- to 6-in., and 6- to 12-in. cultivated and virgin sites from each of the areas sampled did not differ significantly, and the organisms were all classified as *Pseudomonas*.

Figure 1 shows the microscopic morphology of the Gram-negative rods which were found to be 0.75 by 1.5 μ in size when stained with methylene blue. Copious amounts of slime were produced. Part of this appeared to be stained with carbol fuchsin for the cells appeared larger in size when this stain was employed.

Growth on nitrogen-free mannitol agar as demonstrated in Fig. 2, produced small, colorless, circular, convex colonies with entire edges and butyrous centers. Colony growth on sodium benzoate agar was similar to that obtained with mannitol or glucose agar. Samples from the grey-wooded areas, however, produced a dark brown, water-soluble pigment on sodium benzoate agar.

The small coccoid rods grew profusely on nutrient agar producing 2- to 4-mm, entire, circular, white colonies, which tended to turn a light yellow with age. Growth on nutrient agar slants was white and filiform. Inoculation of nutrient broth produced a flocculent growth, whereas peptone water became cloudy and had a slight ring on the surface.

Growth was enhanced by 0.1% nitrate producing 2- to 4-mm colonies with a light yellow tinge. Growth in nitrate glucose broth resulted in both nitrite and gas being formed. The addition of 0.2% glycine to the sugar plus salt media, however, suppressed development of the organisms. Eosin methylene blue agar produced raised, glistening, pink colonies that darkened in the center after 7 to 10 days' incubation.

The isolates were motile under conditions of the hanging drop motility test. Flagellation, as determined by Liefson's flagella stain (6), was polar.

Gelatin was not liquified, nor was indol produced. Litmus milk was unchanged after 14 days. Brilliant green bile broth showed growth but no gas. Neither acid nor gas was produced from a number of carbohydrates and the Voges-Proskauer test for acetyl methyl carbinol was negative.

The lead acetate strip test for H_2S production showed erratic results but Kligler's iron agar demonstrated H_2S production.

The pseudomonads have a greater ability than *Azotobacter* to withstand

TABLE II

Atmospheric nitrogen fixed by *Pseudomonas azotogensis* after 48 hours' incubation in 5 ml of 2% mannitol solution

Location of soils for which organisms were isolated	Nitrogen content		Nitrogen fixed	
	Initial	After incubation	μg/ml	mg/g of mannitol
Youngstown	.220 (.19–.26)*	.307 (.27–.34)*	17.4	0.87
Gleichen	.087 (.08–.10)	.478 (.47–.50)	78	3.91
Halkirk	.028 (.020–.030)	.048 (.030–.070)	4	0.20
Strome	.132 (.11–.16)	.237 (.15–.32)	21	1.05
Airdrie	.158 (.14–.17)	.212 (.18–.25)	11	0.55
Edmonton	.152 (.10–.25)	.465 (.43–.50)	63	3.13
Waskatenau	.113 (.10–.13)	.143 (.11–.17)	6	0.30
Lac La Biche	.218 (.21–.23)	.270 (.19–.32)	10	0.52
Breton	.168 (.13–.18)	.228 (.16–.29)	12	0.60

*Range of four determinations.

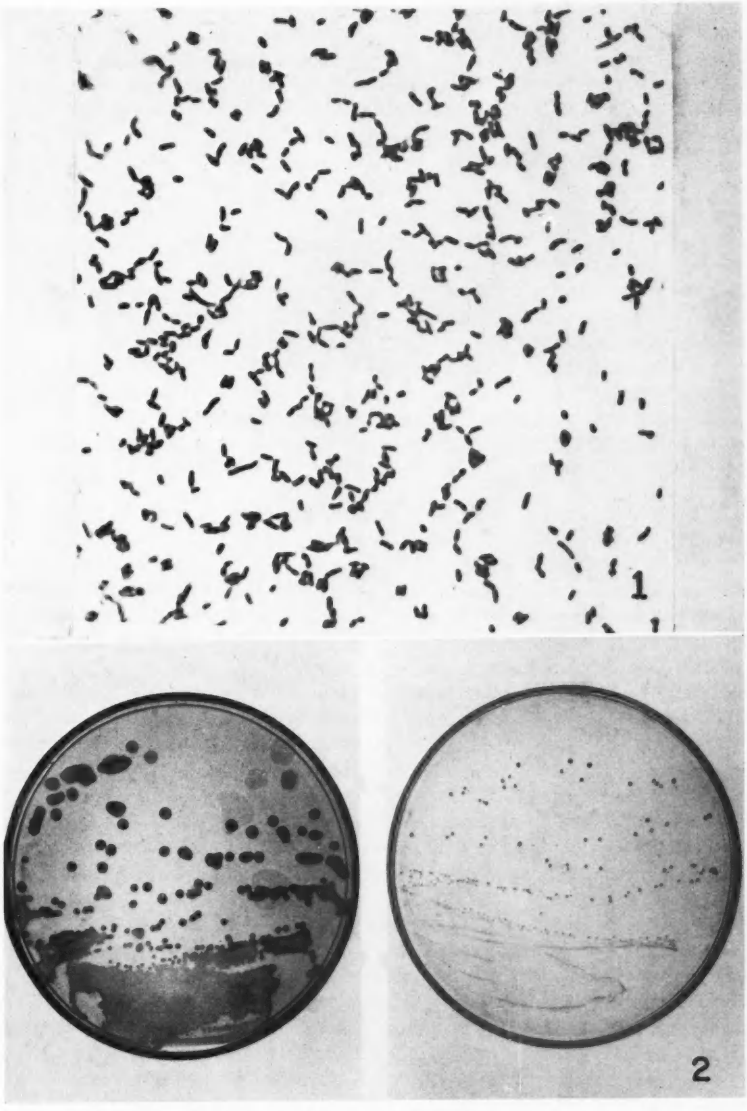

FIG. 1. Microscopic morphology of nitrogen-fixing pseudomonads.
FIG. 2. Growth of nitrogen-fixing organisms on nitrogen-free media; (A) *Azotobacter*, (B) *Pseudomonas*.

acidic pH values. *Azotobacter* did not develop below a pH of 6.2 whereas the growth of the smaller nitrogen-fixing organisms, although scant, extended from pH 4.9 to 7.9.

The optimum growth temperature for *Pseudomonas* was 25° to 28° C and growth could be initiated at temperatures as low as 8° C. The thermal death temperature for a 10-minute exposure was found to lie between 50° and 55° C.

The data obtained from the micro-Kjeldahl determination of the amount of atmospheric nitrogen fixed by isolates from various soils of Alberta are summarized in Table II. Non-symbiotic nitrogen fixation is usually considered to be growth bound. Therefore, visual observations of growth should be a fairly safe criterion for determining the ability of an organism to fix nitrogen. Ascertaining the extent of growth with organisms such as these is, however, difficult because of the extensive amounts of slime produced on nitrogen-free media. This is demonstrated by the variation in the nitrogen content of the different isolates shown in the first column of Table II. The inocula for all the isolates had been adjusted to 15% transmittance at the maximum adsorption wave length of 585 mμ. The results in Table II show that the nitrogen fixed in 5 ml of 2% mannitol varied from 4 to 78 μg/ml. The fixation per gram of mannitol therefore ranged from 0.2 to 3.9 mg N per gram of mannitol. Fixation was statistically significant at or above the 5% level of significance for all the cultures tested.

Nitrogen-fixing Organisms in Saskatchewan Soils

The studies of the non-symbiotic nitrogen-fixing organisms in Alberta soils had been conducted with soils collected in 1954 and 1955. In the spring of 1959 the stored soil samples were again tested for the occurrence of organisms capable of developing on a nitrogen-free medium. *Azotobacter* had not survived storage, but the small Gram-negative pseudomonads were still abundant.

To verify and extend the findings obtained from Alberta soils, samples from virgin and cultivated sites of the brown, dark brown, and black soil zones of Saskatchewan were collected and studied. Soils from 16 sites varied in texture from a fine sandy loam to a clay and had pH values of 5.5 to 7.6.

Azotobacter were found in three of the soils studied. A brown solodized-solonetz clay loam having a pH value of 7.6 mapped as a member of the Echo Association, a silty clay loam (Elstow pH 6.4) from the dark brown soil zone, and a similar orthic member (Blaine Lake pH 6.6) from the black soil zone were the only soils from which it was possible to isolate these nitrogen-fixing organisms.

The study of Alberta soils indicated that *Azotobacter* were associated with irrigated soils mainly. Only one irrigated soil was sampled in Saskatchewan. Plots at the South Saskatchewan predevelopment station at Outlook had been irrigated for 10 years but *Azotobacter* were not present in these soils.

Liquid cultures and dust-plate techniques indicated that the soils of Saskatchewan all contained other organisms capable of growing on media which did not contain added nitrogen. The colonies on nitrogen-free glucose agar, some of which reached a size of 5 to 6 mm, were opalescent, raised, viscous with smooth edges. Microscopic observations showed a variety of organisms growing in conjunction with small, hard to stain, Gram-negative rods similar

to those isolated from Alberta soils. Further isolation procedures separated the small rods which were probably the source of nitrogen for the other contaminating organisms. These contaminants were often yeasts and spore-forming rods having a decided tendency to turn Gram-negative with age.

Discussion

The characteristics of the *Azotobacter* found in Alberta and Saskatchewan soils agree with those described in Bergey's Manual (2) by Jensen (4) as those belonging to *A. chroococcum*. The part played by these organisms in the nitrogen economy of the prairie soils is, however, questionable. In Alberta, *Azotobacter* were found primarily in irrigated soils and in Saskatchewan only 3 of 16 dry-land sites sampled contained these nitrogen-fixing organisms. Jensen (4) questioned the ability of *Azotobacter* to add substantial amounts of nitrogen to the soil under field conditions. Their importance even when present in the soils is probably negligible.

Aerobic organisms other than *Azotobacter* have been often described as capable of growing on nitrogen-free media (16, 7, 5). The Kjeldahl method of determining nitrogen in media inoculated with small numbers of the organisms has, however, failed to show conclusive evidence of fixation. Ross (14) in a series of biological studies of some Tussock grassland soils in New Zealand isolated 11 strains of *Bacterium radiobacter* and three strains of organisms described as *Pseudomonas* capable of growth on media containing no added nitrogen. Kjeldahl determination of nitrogen content of the cultures after 28 days' incubation demonstrated that nitrogen fixation was insignificant under the conditions utilized.

More refined techniques have, however, demonstrated that the ability to fix nitrogen is fairly widespread. Proctor and Wilson (12, 13) found that six randomly selected strains of *Pseudomonas* and eight of *Achromobacter* fixed nitrogen. An inducible enzyme system appeared to be involved in the fixation of 1.1 to 4.3 mg N per gram of carbohydrate.

The Gram-negative, nitrogen-fixing rods isolated in this study are similar to those described by Voets and Debacher (17) for a nitrogen-fixing *Pseudomonas* which they called *Pseudomonas azotogensis*. The size, slime production, staining reactions, biochemical properties, and growth on various media were all similar. Anderson (1) has also isolated a similar nitrogen-fixing *Pseudomonas* to which he assigned the species name *azotocolligans*. The two species designations may be reconcilable. They appear to be similar in that they fix approximately 1 to 4 mg of atmospheric nitrogen per gram of mannitol, and have identical characteristics except for variations in H_2S formation and pigment production.

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STUDIES ON THE NUTRITION OF PHYTOPHTHORA CRYPTOGEA[1]

D. C. ERWIN[2] AND H. KATZNELSON[3]

Abstract

Phytophthora cryptogea, cause of a root disease of alfalfa, grew well over a wide range of pH values (5.6–7.2) on a synthetic medium containing sucrose as the carbon source. The fungus also grew well on glucose and soluble starch. L-Asparagine was the most favorable source of nitrogen, but failed to support growth of a variant obtained from a single germinated oospore. Low concentrations of $CaCl_2.2H_2O$ stimulated growth of *P. cryptogea*, *P. drechsleri*, *P. parasitica*, and *P. boehmeriae*. The minimal concentration of thiamine for growth was between 12 and 25 μg/liter. Soil extract, representing 50% or 90% of the volume of the synthetic medium, did not supply the thiamine requirement or stimulate growth of the fungus. Alfalfa root extract, alone or added to the synthetic medium, not only satisfied the thiamine requirement of the fungus but produced about 30% more mycelial growth than the complete synthetic medium.

Introduction

During the course of an investigation of the biology of *Phytophthora cryptogea* Pethyb. & Laff., cause of a root disease of alfalfa (7), a synthetic medium was needed to determine the presence or absence in soil and roots of certain nutrients necessary for growth of the fungus. Some of the work leading to the development of such a medium, and including especially the effects of calcium and minimal concentrations of thiamine on growth of *P. cryptogea*, are reported. Results of bioassays of soil and alfalfa root extracts for thiamine and other nutrients are also presented.

Methods

Medium

Unless otherwise designated the composition of the basal synthetic medium was: anhydrous L-asparagine (in some experiments freed of water-soluble vitamins by precipitation in ethyl alcohol), 2.0 g; $MgSO_4.7H_2O$, 0.1 g; $FeSO_4.7H_2O$, 0.001 g; KH_2PO_4, 0.43 g; K_2HPO_4, 0.30 g; thiamine HCl, varied from 25 to 1000 μg; 1 ml of a minor element mixture which provided, in the final solution, 1 p.p.m. of Zn ($ZnSO_4.7H_2O$) and 0.02 p.p.m. of Cu ($CuSO_4.5H_2O$), Mo ($NaMoO_4.2H_2O$), and Mn ($MnCl_2 \cdot 4H_2O$) (15); sucrose, 15 g (added aseptically after steam-sterilization); $CaCl_2.2H_2O$, 0.01 to 0.1 g (added with the carbohydrate source, since a precipitate formed when the salt was added prior to autoclaving); and double-distilled water to 1 liter. The pH of the medium was adjusted with NaOH to 6.3–6.5. For critical tests concerned with the determination of the minimal concentration of thiamine required for growth, a stock solution of thiamine, dried over $CaSO_4$, was prepared in 25% ethyl alcohol acidified to pH 3.5 with HCl (1).

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Inoculum

A hyphal tip isolate of *Phytophthora cryptogea* was used in all experiments except where designated otherwise. Each flask was inoculated with a 3-mm disk from the advancing margin of a colony on the synthetic agar medium containing not over 50 μg thiamine per liter. Since little or no growth developed on a thiamine-free medium, the small amount of this essential vitamin in the inoculum disk was not considered an important source of error. For initial tests with other vitamins, a small piece of aerial mycelium was transferred to the medium by means of a metal probe. To prevent contamination of the medium with vitamins that might be present in cotton plugs, flasks were covered with aluminum caps in most tests.

Growth of the Fungus

Pyrex glassware was either boiled or autoclaved in a laboratory detergent, and rinsed with distilled water. The fungus was grown in 50-ml Erlenmeyer flasks containing 10 ml of medium, although in some of the early tests 125-ml Erlenmeyer flasks containing 25 ml of medium were used.

Since the fungus tended to grow erratically in a tight ball when agitation of the medium (80 excursions per minute on a laboratory shaker) was begun immediately after inoculation, the fungus was incubated for the first 2 days without shaking and for 6–8 days thereafter on the shaker at 25°–27° C. Mycelial mats were washed thoroughly on a 200-mesh copper wire screen to which suction was applied, blotted on filter paper, and dried at 60°–70° C for 24 hours. Dry weights and final pH values of the media represent averages of at least four replications.

Results

Growth Rate

Growth of the fungus was more rapid when the medium in the flasks was shaken. In a typical test the final pH and dry weight of mycelium, respectively, were as follows: shake culture, pH 6.8 and 104 mg, and still culture, pH 5.0 and 62 mg.

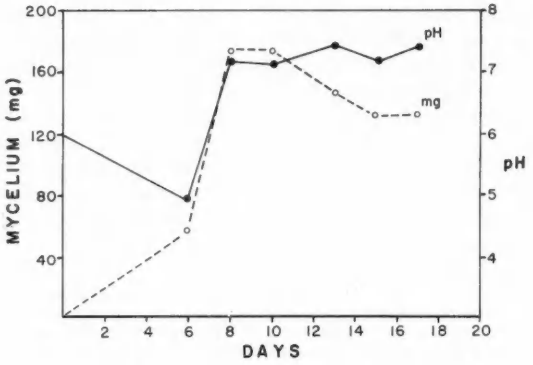

FIG. 1. Growth of *Phytophthora cryptogea* on a synthetic medium containing sucrose and L-asparagine with concomitant change in pH.

In experiments to determine the optimum time for harvesting mycelium growth, the fungus reached a maximum (Fig. 1) 8–10 days after inoculation. Longer incubation resulted in a decrease in dry weight, indicating autolysis. In another experiment, incubation for a second 10-day period resulted in a 27% loss in weight (Table I).

TABLE I

Comparison of the effects of 10 g/liter of glucose, sucrose, and soluble starch on growth of *P. cryptogea* at two concentrations of L-asparagine after two periods of incubation

		Incubation period (days)			
		10		20	
Carbohydrate	L-Asparagine g/liter	Mycelial weight* (mg)	Final pH	Mycelial weight (mg)	Final pH
Glucose	2	124	7.8	91	7.4
	4	122	7.8	99	7.5
Sucrose	2	126	7.8	103	7.5
	4	125	7.8	100	7.3
Starch	2	107	7.8	80	7.4
	4	103	7.8	89	7.5
None	4	8	7.7	—	—

*Dried 24 hours at 60–70° C.

Growth on Glucose, Sucrose, and Starch

Growth on sucrose and glucose at two levels of L-asparagine was approximately the same and about 14% greater than on starch (Table I). L-Asparagine was not utilized more than slightly as a source of carbohydrate.

Results shown in Table I indicated that sucrose was a satisfactory source of carbohydrate. The optimum concentration of sucrose for growth in subsequent experiments was between 15 and 25 g/liter. Data for both 10- and 20-day incubation periods are summarized in Table II. Final pH values were usually lower at the higher concentrations of sucrose.

Nitrogen Utilization

In experiments subsequent to that shown in Table II growth was essentially the same at 1, 2, and 4 g/liter of L-asparagine. The effects of several different

TABLE II

Comparison of growth of *Phytophthora cryptogea* at several concentrations of sucrose

	Incubation period			
	10 days		20 days	
Sucrose (g/liter)	Dry weight (mg)	Final pH	Dry weight (mg)	Final pH
5	32	7.1	—	—
15	111	5.5	—	—
25	124	4.9	131	4.6
45	111	4.8	121	4.4

sources of nitrogen were each compared at a concentration of 212 mg nitrogen (equal to 1 g/liter of L-asparagine). Since Newton (17) showed that *Phytophthora parasitica* Dastur utilized DL-alanine but not D-alanine, the concentration of DL forms of amino acids was doubled.

Since mycelial weights usually varied between experiments, growth on each nitrogen source was compared to L-asparagine and vitamin-free casein hydrolyzate (Nutritional Biochemical Co., Cleveland, Ohio) in each experiment. Results are shown in Table III. Although excellent growth on L-histidine, L-alanine, glycine, DL-threonine, DL-serine, and L-arginine was obtained, the mycelial weights were always greatest on either L-asparagine or casein hydrolyzate. In a previous experiment L-proline did not support growth.

TABLE III

Growth response of *Phytophthora cryptogea* to several sources of nitrogen

Nitrogen source	Mycelial weight (mg)	Final pH
L-Asparagine	109	5.7
Casein hydrolyzate	104	5.1
L-Histidine	79	4.3
L-Alanine	70	4.9
Glycine	66	4.7
DL-Threonine	61	4.7
DL-Serine	57	4.8
L-Arginine	50	4.1
L-Aspartic acid	28	5.4
L-Valine	28	4.8
DL-Ornithine	24	3.9
L-Cystine	20	5.0
L-Glutamic acid	18	5.8
L-Lysine HCl	14	4.1
L-Leucine	6	5.5
KNO_3	6	5.5
L-Methionine	5	4.4
L-Tyrosine	2	6.2
DL-Phenylalanine	2	4.4
$(NH_4)_2SO_4$	0	5.6

When $(NH_4)_2SO_4$, which did not support growth of the fungus (Table III), was included in a medium containing L-asparagine, mycelial weight was reduced from 98 mg to 74 mg by addition of 0.4 g/liter but not by 0.1 or 0.2 g/liter. At the highest concentration of $(NH_4)_2SO_4$ final pH of the medium was reduced to 4.9 compared to 7.0 in its absence.

During a study of oospore germination of the isolate used in this study, six colonies from single oospores were obtained. Three of these did not grow on L-asparagine but grew well on vitamin-free casein hydrolyzate as a source of nitrogen. One of the latter isolates did not grow on any of the nitrogen sources listed in Table III except casein hydrolyzate.

Effect of pH on Growth

When the synthetic medium was adjusted to the desired pH and buffered with a 0.005 *M* KH_2PO_4–K_2HPO_4 combination at ratios required for each pH value, maximum growth (187–198 mg) occurred at pH 5.6 to 7.2, but much less (47 mg) at pH 5.1 and virtually none at pH 4.9 and 7.8.

When several concentrations of a KH_2PO_4–K_2HPO_4 buffer were tested at pH 6.6, growth was approximately the same at 0.0025 to 0.01 *M* concentrations of buffer (82–88 mg) but at a 0.015 *M* concentration, growth was reduced to 70 mg. The final pH values at each buffer concentration were as follows: 0.0025 *M*, 7.8; 0.005 *M*, 7.4; 0.0075 *M*, 6.9; 0.01 *M*, 6.6; and 0.015 *M*, 5.4.

Effect of Calcium on Growth of Phytophthora spp.

The results of three experiments showed a stimulation of growth by low concentrations of $CaCl_2.2H_2O$. The data in Table IV are typical of those obtained in these tests.

TABLE IV

Effect of adding $CaCl_2.2H_2O$ to media containing 0, 0.25, and 0.50 g/liter of the disodium salt of ethylene-diamine tetraacetic acid on growth of *Phytophthora cryptogea*

$CaCl_2.2H_2O$ (g/liter)	Mycelial wt. and final pH of the medium					
	Na_2EDTA (g/liter)					
	0		0.025		0.050	
	mg	pH	mg	pH	mg	pH
0.0	60	6.0	12	4.8	0	6.0
0.005	93	7.0	78	5.3	12	5.0
0.01	94	7.4	72	5.9	58	4.9
0.05	98	7.3	100	7.2	104	7.2
0.10	94	7.3	97	7.1	95	7.2
0.20	94	7.3	96	7.1	98	7.0

To determine if the growth-stimulating effect of $CaCl_2.2H_2O$ was due to calcium and not to chloride, the effects of CaO, $CaSO_4$, and $CaCl_2.2H_2O$ were compared. The concentration of calcium (2.7 mg/liter) used was equal to that in $CaCl_2.2H_2O$ at 0.01 g/liter. Approximately 100 mg of mycelium were obtained from each of the media receiving a calcium salt but only 57 mg of mycelium were obtained from the medium not receiving calcium.

Ethylene–diamine tetraacetic acid (EDTA), a metal-chelating agent considered to be biologically inert (10), was reported by Reischer (19) to be useful in studying the inorganic nutrition of species in the family Saprolegniaceae: she stated that "tight chelation" (by EDTA) allowed a large reservoir of metallic ions which became available gradually by mass action, and also provided a lower concentration of complex-forming contaminants in chemicals, glassware, or inocula.

Since EDTA chelates calcium readily (16), growth in media which contained a series of concentrations of $CaCl_2.2H_2O$ was compared with and without the disodium salt of EDTA (Na_2EDTA) at two concentrations.

The results shown in Table IV are representative of two experiments. In the presence of Na_2EDTA, little or no growth occurred when $CaCl_2.2H_2O$ was omitted, but with increasing concentrations of $CaCl_2.2H_2O$ growth increased to the maximum. In the absence of Na_2EDTA growth in the calcium-deficient medium was about 65% of that in media to which calcium had been added.

Although the depression of growth by Na_2EDTA at low concentrations and in the absence of $CaCl_2.2H_2O$ might have been due to chelation of other essential metals, the data suggest the possibility that a small residual quantity of calcium might exist as a contaminant of the other components of the medium. Further indication of this was obtained from a preliminary spectrographic analysis of the complete medium which showed a low concentration of calcium in the medium to which $CaCl_2.2H_2O$ had not been added.

Further tests showed that $CaCl_2.2H_2O$ (0.1 g/liter) increased growth of another isolate of *Phytophthora cryptogea* from alfalfa from 43 to 72 mg; *P. drechsleri* Tuck., pathogen of safflower, from 36 to 53 mg in one experiment, and from 58 to 103 mg in another; *P. boehmeriae* Sawada, from 36 to 48 mg; *P. parasitica* Dastur, pathogen of *Citrus* spp., from 48 to 68 mg.

Effect of Vitamins on Growth

To determine the vitamin requirements of *Phytophthora cryptogea*, several of those known to be necessary for some microorganisms were placed in two groups after the method of Lochhead and Burton (14). Group A included, in μg/liter, calcium pantothenate 500, biotin 1.0, vitamin B_{12} 2.0, folic acid 100, and nicotinic acid 500. Group B included riboflavin 500, pyridoxine 500, pyridoxal 500, pyridoxamine 100, and *p*-aminobenzoic acid 500. Thiamine at 500 μg/liter was tested with and without both group A and B combined or separately. Yeast extracts from three sources (Difco, Nutritional Biochemical Co., and British Drug House) were also tested (at 1 g/liter) with and without thiamine. The control medium contained no thiamine.

Growth of the fungus was essentially the same (79–89 mg, final pH 7.3) in all flasks which contained either thiamine or yeast extract. In the medium which did not contain thiamine or yeast extract, the fungus produced only 1–3 mg of mycelium and the pH of the medium consistently dropped to 4.7–5.1. The data indicated that vitamins other than thiamine were not required by the fungus.

The minimal concentration of thiamine for near-maximum growth appeared to be between 12 and 25 μg/liter (Fig. 2). Increase in concentration of thiamine from 25 to 100 μg/liter usually resulted in only a slight increase in growth

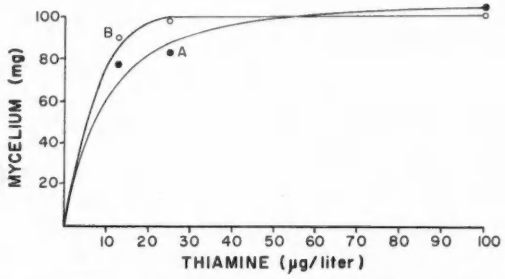

FIG. 2. Effect of concentrations of thiamine on mycelial weight of two isolates of *Phytophthora cryptogea*. A. Typical response of the isolate used in these studies. B. Response of another isolate.

(Fig. 2,A). One isolate, however, grew as well at 12 μg as at 100 μg/liter (Fig. 2,B). In other experiments, increasing concentrations of thiamine up to 500 and 1000 μg/liter had no effect on growth.

Growth of the Fungus on Soil Extract

Since *Phytophthora cryptogea* is a soil-borne fungus the nutritional effects of soil extract on growth of the fungus were studied. Soil extract was prepared by autoclaving for 20 minutes a 1:1 soil–water suspension and filtering. To determine if thiamine was supplied by the extract, it was added to thiamine-deficient synthetic media, and to determine a possible toxic effect, it was also added to thiamine-containing media. Controls consisted of thiamine-deficient and thiamine-containing synthetic media.

The results of these experiments showed that soil extract and soil were poor sources of nutrients for the fungus, but not toxic. Data for one experiment, which was typical of others, are given in Table V. Addition of soil extract as 50% or, in other experiments, as 90% of the volume of the synthetic medium had no stimulatory effect on growth either in the presence or absence of thiamine.

TABLE V

Effect of addition of soil extract (SE) to a synthetic medium on growth of *Phytophthora cryptogea*

Treatment	Thiamine (75 μg/liter)	Mycelial wt. (mg)	Final pH
SE (50%) + synthetic medium	+	80	7.8
SE (50%) + synthetic medium	0	2	4.7
− synthetic medium	+	84	8.0
− synthetic medium	0	2	4.6

In another experiment soil extract plus sucrose and thiamine supported no growth of the fungus. Soil extract plus sucrose, L-asparagine, and thiamine produced only 13 mg of mycelium, compared to 80 mg in the complete synthetic medium. Extract from a soil amended with alfalfa foliage (2% by weight) and incubated for 22 days likewise did not substitute for thiamine in the synthetic medium. Growth on the thiamine-containing medium containing soil extract was similar to that on the complete synthetic medium.

Since the presence of thiamine has been demonstrated in soils (11, 22), perhaps either the soil solution was not concentrated sufficiently or thiamine was adsorbed by soil. To test the fate of synthetic thiamine in soil, 1000 g of soil were amended with 75 μg of the vitamin. When soil extract from this soil was added to a thiamine-deficient medium, only 11 mg of mycelium developed, compared to 90 mg on the medium containing soil extract plus an excess of thiamine. It appeared that a large part of the thiamine could not be extracted from soil by the method used.

In several other tests soil was added directly to thiamine-deficient and complete synthetic media at concentrations of 1 to 100 g/liter. Less than 3 mg of mycelium were harvested from each of the thiamine-deficient media, compared to 70 to 75 mg of mycelium from those containing thiamine.

Effect of Alfalfa Root Extract on Growth

To determine the effect of alfalfa root extract on growth of the fungus root extract (RE) was prepared by soaking 320 g of air-dried alfalfa tap roots in 1000 ml of distilled water for 3 hours. The media were as follows: (1) 50% RE alone, (2) 50% RE in double strength synthetic medium, (3) 50% RE plus sucrose, (4) 50% RE plus L-asparagine, (5) 90% RE alone, and (6) the synthetic medium (control). Portions of all media except medium 4 were tested with and without thiamine (100 μg/liter).

The data in Table VI show that RE contained all of the components necessary for maximum growth of the fungus since neither the addition of sucrose, L-asparagine, thiamine, nor the complete medium increased the growth above that in RE alone. The weight of mycelium was also greater in RE alone than in the synthetic medium, which indicated that the synthetic medium either did not satisfy all of the nutritional requirements of the fungus or that there was an imbalance of nutrients. The reason for the low yield of mycelium and final pH of the medium in the 50% RE plus the complete medium (minus thiamine) is not apparent, since 100 mg of growth were recorded for 50% RE alone, without thiamine.

TABLE VI

Effect of alfalfa root extract on growth of *Phytophthora cryptogea*

% concentration of root extract in the medium	Component of medium added to root extract	μg/liter of thiamine added to medium	Mycelial weight (mg)	Final pH
50	None	100	109	6.4
		0	100	5.7
50	Sucrose	100	106	6.5
		0	94	5.9
50	L-Asparagine	100	95	7.8
		—		
50	L-Asparagine and sucrose	100	95	7.8
		0	92	7.0
50	Complete medium	100	98	8.0
		0	38	4.4
90	None	100	102	7.7
		0	96	5.8
0	None, complete medium	100	84	8.0
		0	2	4.6

Discussion

Since a single oospore variant of *Phytophthora cryptogea* failed to utilize any single amino acid but grew well on the complex casein hydrolyzate, it appears that it needs more than one amino acid or lacks the ability to metabolize L-asparagine to other required amino acids. For instance, Close (3) demonstrated several amino acids and two amides in the mycelium of *P. cactorum* (Leb. and Cohn) Schroet., grown with only L-asparagine as a nitrogen source. The failure of the *P. cryptogea* variant to utilize L-asparagine might be a useful genetic marker for further studies.

Steinberg (25) found that calcium was necessary for *Rhizoctonia solani*

Kühn but not for *Fusarium oxysporum* Schlect var. *nicotianae* J. Johnson, both plant pathogens. The growth-stimulating effect of calcium on *Phytophthora cryptogea* adds to the information previously reported by Lopatecki and Newton (15), who reported that $CaSO_4$ stimulated the growth of *P. parasitica*, *P. cactorum*, *P. megasperma*, and *P. erythroseptica*, and by Davies (6), who recently showed an absolute requirement of *P. fragariae* for $CaCl_2$. On the other hand, Hodgson (9) obtained no reduction in growth of *P. infestans* when $CaCl_2$ was omitted from a medium. Christie (4) could detect no growth of *P. cactorum* when calcium salts were added to a medium containing $NaNO_3$ as a nitrogen source but good growth developed when $Ca(NO_3)_2$ was used as a nitrogen source. Comparatively little attention has been given to the role of calcium in studies of the nutrition of *Phytophthora* spp. Since a response has been shown for several species, omission of calcium from a synthetic medium might act as a limiting factor and obscure certain effects of other treatments on growth.

The finding that thiamine is necessary for growth of *P. cryptogea* adds to the already abundant evidence (18, 20, 21) on heterotropism of *Phytophthora* spp. for this vitamin.

Although no studies were made of the effect of thiamine concentration as related to concentration of carbohydrate and nitrogen levels, it appeared that the minimal concentrations necessary for growth of *P. cryptogea* (12–25 μg/liter) were somewhat less than those reported for other *Phytophthora* species except *P. cinnamomi* (24, p. 260).

Although presence of thiamine in soil has been demonstrated (11, 22), in our studies, not enough thiamine to support growth of *P. cryptogea* in vitro could be recovered even when minimal quantities of the vitamin were artificially added previous to soil extraction. This might indicate that thiamine, although water soluble, is partly adsorbed by the soil. Schopfer (24) stated that adsorption of this vitamin by organic materials might be a source of error in bioassays.

Lochhead (13) summarized the literature on vitamin synthesis and destruction by microorganisms in soil and stated that "concentration of a growth factor at any time depends on the balance established between synthetic and destructive agencies." More information on the availability of thiamine in soil might be extremely important for an understanding of the ecology of *P. cryptogea*.

Alfalfa root extract, in contrast to soil extract, contained thiamine as well as all the other nutrients necessary for growth of the fungus. No attempt was made to determine whether thiamine or thiamine-like substance in root extract originated in the rhizosphere or from the plant. However, Lochhead (12) and Cook and Lochhead (5) showed that in the rhizosphere, bacteria which synthesized growth factors (including thiamine) were relatively more abundant than those which required them. This information favored the view that essential growth-promoting substances were produced predominantly "through the synthetic abilities of other organisms" (in the rhizosphere) rather than from root exudates. Thus the rhizosphere may be an even more important habitat for *P. cryptogea* than has been realized heretofore.

Further knowledge concerning the thiamine nutrition of *Phytophthora* might be important in a study of the parasitism of the fungus. Garber (8) recently summarized literature on parasitism of plants and animals by micro-organisms and presented a nutrition-inhibition hypothesis of pathogenicity. Bitancourt and Rossetti (2) reported that thiamine increased the ramification of branches of hyphae of *P. citrophthora* but not elongation. In a later paper they (23) showed, by an auxanographic method, diffusion of thiamine or thiamine-like materials from disks of citrus bark to a synthetic agar medium. Thickness of the mycelial mats was greater in the medium from which bark disks of the susceptible sweet orange had been removed than in the medium from which the resistant sour orange bark disks had been removed. They also postulated another factor "L" found in agar and natural substances which conditioned elongation of hyphae. It appears that more detailed studies of the effects of nutritional factors on the ecology of *Phytophthora* spp. are warranted.

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CHEMICALLY DEFINED MEDIUM FOR GROWTH OF MICROCOCCUS LYSODEIKTICUS[1]

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Abstract

A chemically defined medium for growth of *M. lysodeikticus* is presented. The organism possesses a relatively nonspecific but absolute purine requirement that can best be satisfied by the free base hypoxanthine although adenine also allows some growth. A substitution for hypoxanthine, however, can be made by inosine, adenosine, or adenylic acid, but not by guanosine or guanylic acid. Although biotin stimulates growth, equally good growth occurs using biocytin or biotin-*d*-sulphoxide. Less stimulation is apparent using desthiobiotin, *dl*-oxybiotin, or biotin-*l*-sulphoxide. Although amino acids are necessary for growth, no absolute requirement for a specific amino acid can be demonstrated. The amino acid requirements need to be defined in terms of those amino acids which support good growth in the presence or absence of glutamic acid.

Introduction

Micrococcus lysodeikticus is an important organism because the cell wall of this organism contains large amounts of the substrate of lysozyme. Before studies can proceed relating to cell wall synthesis, utilizing the substrate of lysozyme as an indicator or reference substance, it would be advantageous to be able to grow the organism in chemically defined media.

Feiner, Meyer, and Steinberg (1) attempted to devise a synthetic medium for growth of *Micrococcus lysodeikticus* using three immunologically distinct strains. Growth occurred only when the *Lactobacillus casei* factor (a crude folvite liquor containing folic acid) was present. When they attempted substitution using pure folic acid and other B vitamins, growth did not occur.

Wessman, Allen, and Werkman (5) were able to grow their strain of *M. lysodeikticus* in a synthetic medium containing 16 amino acids, 8 B-vitamins, purines, a pyrimidine, salts, and glucose. They observed that biotin was required for growth.

Wolin and Naylor (6) reported that their strain of *M. lysodeikticus* grew better in a medium consisting only of mineral salts, biotin, glucose, and monosodium glutamate than in complex media.

We have employed the Purdue University strain of *M. lysodeikticus*. To our knowledge, this organism is a transplant from the original strain isolated by Fleming and sent to this country about 14 years ago. It has been utilized in previous studies relating to the action of lysozyme (2, 3, 4). Perhaps different "nutritional" strains exist since this organism will not grow in any of the proposed synthetic media.

It is difficult to define *M. lysodeikticus* since the organism is described in the 6th Edition of Bergey's Manual only as a yellow micrococcus, easily lysed by lysozyme. It is not listed in the 7th Edition of Bergey's Manual.

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Contribution from the Department of Bacteriology, Oklahoma State University, Stillwater, Oklahoma. This work was supported in part by grants from National Institutes of Health, U.S. Public Health Service (E-2530), and the Research Foundation, Oklahoma State University (State Project 91).

Materials and Methods

For inoculation purposes, the organism was grown for 22–24 hours on nutrient agar slants at 37° C, washed twice in 10 ml of 0.85% sterile saline solution, and resuspended in approximately 10 ml of sterile saline. One drop of the washed suspension (O.D. 0.22–0.26) was inoculated into a total volume of 5 ml of the various media formulations. Because aeration is necessary for optimum growth, tubes (17 mm I.D.) were always incubated with shaking either at 25 or 32–35 C.

Basal medium contained, per 5 ml, 1.0 ml of trace mineral salts solution to give the following concentrations per ml of medium: H_3BO_3 0.025 μg, $CaCO_3$ 0.05 μg, $CuSO_4.5HOH$ 0.005 μg, $FeSO_4(NH_4)_2SO_4.6HOH$ 0.25 μg, KI 0.005 μg, $MnSO_4.HOH$ 0.01 μg, MoO_3 0.005 μg, and $ZnSO_4.7HOH$ 0.025 μg (soln. I); 1.0 ml of buffer–salts solution to give the following amounts per ml of medium: NH_4Cl 1.0 mg, K_2HPO_4 2.0 mg, and $MgSO_4.7HOH$ 20 μg (soln. II), and, in most studies, 0.5 ml of "vitamin-free" enzymatically hydrolyzed casein (from Nutritional Biochemicals Corp.). The commercial 5.0% solution was adjusted to pH 7.6–7.8 using solid potassium hydroxide prior to its addition to the medium. Toluene was routinely added to these solutions during storage to prevent contamination. When heat-labile compounds were used, they were sterilized by passage through a sintered-glass filter and added aseptically; otherwise the medium was sterilized by autoclaving 12 minutes at 10 lb pressure.

Results and Discussion

Repeated additions of B vitamins, purines, pyrimidines, carbohydrates, or fatty acids to the basal medium (salt solutions plus casamino acids) failed to elicit growth except in one experiment where some growth occurred in the presence of adenine after extended incubation (0.12 O.D. after 96 hours at 25° C on a rotary shaker). When adenosine was substituted for adenine, growth was improved (0.22 O.D. after 24 hours at 25° C). Attempts were made to obtain increased growth response by adding B vitamins in the presence of adenosine. Biotin stimulated growth at 24 hours and some stimulation was also observed at 48 hours in the presence of inositol. The adenine–ribose relationship was then studied in the presence and absence of biotin.

Equimolar concentrations of adenosine (2.2×10^{-4} *M*), adenine, *d* (−) ribose, and adenine plus ribose were added separately to the basal medium. In the presence of adenosine good growth occurred in 24 hours, whereas in the presence of adenine or adenine plus ribose, growth was still poor after 48 hours; ribose alone could not support growth. Further studies were done using guanosine, guanylic acid, adenylic acid, hypoxanthine, and inosine. Representative results are shown in Table I.

These studies demonstrate that the organism possesses an absolute purine requirement that can best be satisfied by the free base hypoxanthine. Although adenine allows some growth within 48 hours, the growth response is much less than that obtained in the presence of hypoxanthine. The purine requirement is not specific in that adenosine (6-amino purine plus ribose) or inosine (6-keto purine plus ribose) allows growth equivalent to hypoxanthine (6-keto purine).

Response to guanosine (2-amino-6-keto purine plus ribose) was extremely poor. Pyrimidines (uracil or cytosine) could not substitute for the purines. Orotic acid (a pyrimidine precursor) also failed to allow growth.

TABLE I

Influence of purines and purine nucleosides and nucleotides on growth of *Micrococcus lysodeikticus**

Compound added†	Optical density‡	
	24 hours	48 hours
None	0.01	0.015
Adenine sulphate	0.045	0.08
Adenosine	0.775	1.35
Adenylic acid	0.52	0.90
Inosine	0.85	1.35
Hypoxanthine	0.775	1.35
Guanosine	0.01	0.015
Guanylic acid	0.01	0.02

*All tubes contained basal medium plus biotin (50 μg/100 ml).
†All compounds tested at 2.2×10^{-4} *M* concentration.
‡Measured at 535 mμ against a distilled water blank after growth at 34° C on a reciprocating shaker (100 strokes/minute).

Biotin requirements were also defined. Representative data are included in Table II. Although biotin is only stimulatory, this requirement is only relatively specific since biotin, biotin-*d*-sulphoxide, or biocytin allowed almost equal growth response. Desthiobiotin, *dl*-oxybiotin, and biotin-*l*-sulphoxide, although also capable of stimulating growth, were inferior to biotin in this respect. Biotin could not be replaced by valeric, pimelic, or oleic acids at equimolar or at 50 times the biotin concentration. At 50 times concentration, oleate was toxic.

Inositol stimulation was also studied since this vitamin can exist in hydroxy

TABLE II

Effect of various forms of biotin, inositol, and fatty acids on growth of *Micrococcus lysodeikticus**

Compound added†	Optical density‡
None	0.21
Biocytin	0.49
Biotin-*d*-sulphoxide	0.48
Biotin	0.48
Desthiobiotin	0.40
dl-Oxybiotin	0.35
Biotin-*l*-sulphoxide	0.27
Valeric acid	0.20
Pimelic acid	0.16
Sodium oleate	0.15
Inositol	0.25

*All tubes contained the following per 100 ml: K_2HPO_4 200 mg, NH_4Cl 100 mg, $MgSO_4$ 2.0 mg, inosine 6.0 mg, L-phenylalanine 40 mg, L-tyrosine 100.8 mg, L-glutamic acid 358.4 mg.
†Concentration of biotin compounds 2.04×10^{-4} *M*, valeric acid 20.84 μg/100 ml, pimelic acid 32.6 μg/100 ml, sodium oleate 57.6 μg/100 ml, and inositol 100 μg/100 ml.
‡Measured at 535 mμ against a distilled water blank after growth at 34° C for 29 hours on a reciprocal shaker having 100 strokes/minute.

(inositol) or phosphate (phytic acid) form in nature. Although slight stimulation is apparent in 29 hours in the presence of inositol, no stimulation occurs in the presence of phytic acid.

Replacement of casamino acids by synthetic mixtures of amino acids was attempted. By eliminating amino acids singly or in groups, a mixture of 10 amino acids was chosen which supported good growth; however, a longer lag period occurs in the presence of the synthetic amino acid mixture (Table III).

After the organic needs were defined, the mineral solutions were studied to ascertain if they were required. All of the compounds in solution II are necessary for growth.

TABLE III

Comparison of growth in media containing casamino acids or the 10 amino acid mixture

Tube No.*	Content	Optical density	
		24 hours	48 hours
1	Casamino acids	0.78	1.3
2	10 amino acid mixture†	0.42	1.3

*Both series contained mineral salts, biotin (50.0 μg/100 ml), and adenosine (10.0 mg/100 ml).
†Concentration of amino acids per 100 ml medium: DL-isoleucine 204 mg, L-leucine 147 mg, L-proline 180 mg, L-glutamic acid 358.4 mg, DL-phenylalanine 80 mg, L-tyrosine 100.8 mg, L-arginine 65.6 mg, L-cysteine 48 mg, L-methionine 27 mg, DL-lysine 131 mg.

Solution I can be left out of the medium without an appreciable decrease in growth. It would appear that these mineral salts are not needed, but, more probably, sufficient amounts of them contaminate other chemicals used in the formulation. At times, lack of growth or poor growth has been traced to an apparent iron requirement. If iron is added as ferrous ammonium sulphate prior to autoclaving of the entire medium, growth always occurs; however, a precipitate forms which interferes with optical density measurements. Addition of ethylene diamine tetraacetic acid (EDTA) as an iron chelate does not sufficiently reduce the precipitate. Although precipitate formation does not occur, reduced growth response is apparent when coprogen, hemin, cytochrome *c*, or ferrichrome (1 μg/ml) are used as substitutes for ferrous ammonium sulphate. Therefore, although a requirement for iron is evident, the requirement is not always apparent since in most instances there appears to be enough iron contamination of materials such as phosphates, sodium chloride, etc., to allow growth of the organism. To maintain more constant conditions in routine work, iron has been added to the medium after first autoclaving a solution of ferrous ammonium sulphate containing 2.5 mg/ml, allowing the precipitate to form, and then aseptically adding one drop of the clear portion per 5 ml of autoclaved media.

Although all synthetic formulations for this organism employ glucose, the essentiality of glucose for growth of *M. lysodeikticus* has never been demonstrated. During our early studies, it had been noted that glucose was not necessary for initiation of growth. Because it is well established that an organism can, in many instances, utilize fatty acids resulting from the breakdown of glucose as primary sources of carbon and energy, fatty acids or their

salts were tested to determine if they could decrease lag time. Negative results were obtained with all compounds tested (formic, malic, or succinic acids; potassium fumarate; or sodium salts of pyruvic, butyric, and acetic acids).

Continued study of the amino acid nutrition has shown that all 10 of the amino acids studied need not be present for good growth response by this organism. Cysteine, leucine, isoleucine, proline, methionine, and lysine can be omitted as a group, but growth is impaired to some extent. Presence of any or all of these amino acids appears to stimulate growth during the first 24 hours. At least two and probably three amino acids should be present for what may be considered good growth response in 36 hours (Table IV).

TABLE IV

Response of *M. lysodeikticus* to various combinations of four amino acids*

Amino acids†				Optical density‡	
Arginine	Phenylalanine	Tyrosine	Glutamic acid	24 hours	36 hours
+	−	−	−	0.01	0.01
−	+	−	−	0.04	0.05
−	−	+	−	0.06	0.08
−	−	−	+	0 05	0.08
+	+	−	−	0.05	0.05
+	−	+	−	0.10	0.34
+	−	−	+	0.06	0.09
−	+	+	−	0.08	0.23
−	−	+	+	0.08	0.18
−	+	−	+	0.09	0.67
−	+	+	+	0.10	0.74
+	+	−	+	0.10	0.60
+	−	+	+	0.12	0.65
+	+	+	−	0.09	0.30
+	+	+	+	0.51	1.40
−	−	−	−	0.02	0.02

*All tubes contained salt solution II, biotin (50 μg/100 ml), inosine (6.0 mg/100 ml), and iron solution as explained in the text.

†Concentration of amino acids per 100 ml medium: L-arginine 65.6 mg, L-glutamic acid 358.4 mg, L-phenylalanine 40 mg, and L-tyrosine 100.8 mg.

‡Measured at 535 mμ against a distilled water blank. Cultures incubated on a reciprocating shaker (100 strokes/minute) at 34° C.

These data show that no one amino acid will allow more than very minimal growth. Although two amino acids allow fairly good growth (e.g., phenylalanine and tyrosine; tyrosine and arginine), none appears to be absolutely required since the amino acids can be interchanged and, also, glutamic acid will always increase growth response. For these reasons, no statement can be made regarding essentiality of amino acids and the requirements need to be defined in terms of those amino acids which stimulate growth in the presence or absence of glutamic acid. Apparently, the bulk of amino acid synthesis in this organism can occur using glutamic acid as a precursor substance; however, glutamic acid alone will not satisfy the amino acid requirements. The aromatic amino acids phenylalanine and tyrosine appear to be essential for good growth in 36 hours.

In further media manipulations, the combination of phenylalanine, tyrosine, and glutamic acid has given satisfactory and reproducible growth responses.

Although glutamine can substitute for glutamic acid, chromatography of glutamine that we have used showed that at least four other ninhydrin-positive compounds were present as impurities.

A defined medium is presented in Table V. This medium has been used in routine work and gives reproducible growth responses.

TABLE V

Defined medium for growth of *Micrococcus lysodeikticus**

Compound added	Amount used per 100 ml medium
Biotin	50 μg
Inosine	6.0 mg
L-Glutamic acid	358.0 mg
L-Phenylalanine	40.0 mg
L-Tyrosine	100.0 mg
NH_4Cl	100.0 mg
K_2HPO_4	200.0 mg
$MgSO_4.7HOH$	2.0 mg
$FeSO_4(NH_4)_2SO_4.6HOH$ (as explained in text)†	

*pH is adjusted to 7.6–7.8 using solid KOH prior to autoclaving.
†Also see text for substitutions and (or) additions involving biotin, inosine, and amino acids.

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DEGRADATION OF RUTIN BY ASPERGILLUS FLAVUS. FACTORS AFFECTING PRODUCTION OF THE ENZYME SYSTEM[1]

D. W. S. WESTLAKE AND F. J. SIMPSON

Abstract

A synthetic medium containing 0.4% rutin, 0.3% $(NH_4)_2HPO_4$, 0.1% KH_2PO_4, 0.1% $MgCl_2.6H_2O$, and 0.8% $(NH_4)_2SO_4$ was developed for the production of the extracellular enzyme system that degrades rutin. The total amount of the enzyme system produced is approximately the same between 25 and 35° C but the rate is more rapid at the higher temperature. Aeration is necessary both for synthesis and for excretion into the medium, excretion apparently being more sensitive than synthesis. Magnesium is required for growth and sulphate for release of the enzyme system into the medium. Calcium carbonate depresses both growth and production. The medium has been successfully employed for production in 5-gallon fermentors of the extracellular enzymes that degrade rutin.

Introduction

Several strains of *Aspergillus flavus* produce extracellular enzymes capable of degrading rutin to water-soluble products (15). The initial step in this reaction is postulated as being the cleavage of the glycosidic bond followed by splitting of the γ-pyrone ring between carbon-2 and -3 and release of carbon monoxide from carbon-3 (12). The final step in the sequence is believed to be the hydrolysis of the depside of protocatechuic acid and phloroglucinol carboxylic acid. This paper described studies undertaken to develop a synthetic medium that would permit a high degree of synthesis and excretion of the enzyme system and thus facilitate the purification and separation of the enzymes involved in the degradation.

Methods

Microbiological

Aspergillus flavus (PRL 1805), which produces an extracellular enzyme system capable of degrading rutin to water-soluble products, was selected for this study as it produced culture filtrates of high activity. Stock cultures of this organism were maintained on agar slants (15).

The initial medium used for production of the enzyme system consisted of 0.2% rutin, 0.15% $(NH_4)_2HPO_4$, 0.05% KH_2PO_4, 0.20% N-Z amine, salts (100 mg $MgSO_4.7H_2O$; 1.0 mg each of $ZnSO_4.7H_2O$ and $FeSO_4.7H_2O$; 0.75 mg $MnSO_4.H_2O$ and 0.05 mg $CuSO_4.5H_2O$ per liter), and vitamins (500 μg each of thiamine hydrochloride, riboflavin, calcium pantothenate, and niacin; 100 μg each of pyridoxal, para-aminobenzoic acid, and inositol; 10 μg of folic acid and 4 μg of biotin per liter) (15). The rutin was autoclaved separately with water and the remaining ingredients prepared at 5 times the final concentration, sterilized, and added aseptically to the flasks prior to inoculation. Unless

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otherwise stated, 25 ml of medium was used per 250-ml Erlenmeyer flask. The inoculum, which consisted of 0.5 ml of a 48-hour-old culture, was prepared by inoculating 25 ml of medium with a suspension of spores and then homogenizing the resultant growth for 30 seconds in a sterile Waring Blendor prior to use. The cultures were incubated for 72 hours at 30° C on a Gump rotary shaker (110 r.p.m., $1\frac{1}{4}$ in. eccentricity).

Enzymatic

Quantitative studies were made on culture filtrates prepared as previously described (15), and in addition, the enzyme bound in the mycelium was determined using mycelial suspensions prepared by grinding air-dried mycelium in 5 ml of water with a mortar and pestle.

Enzymatic activity was estimated by incubating a suitable portion (usually 2 ml) of filtrate or mycelial suspension with 2 ml of a 0.1 *M* citric acid – 0.2 *M* Na_2HPO_4 buffer solution, pH 6.5, and 40 mg of rutin in a test tube of 25-mm diameter on a rotary shaker (180 r.p.m., 1 in. eccentricity) for 3 hours at 30° C (total volume 4 ml). The reaction mixture was then diluted with ethanol, which stopped the enzymatic reaction and dissolved the remaining rutin. A suitable aliquot was taken and analyzed for residual rutin. One unit of enzyme was defined as the amount required to degrade 1 mg of rutin in 3 hours at 30° C under the standard conditions described above. This method, since it measures both the rutin and quercetin, which react with aluminum chloride, measures the over-all rate of the enzymes in the system, that is (*a*) the glycosidase, (*b*) the enzyme(s) that break the γ-pyrone ring and release carbon monoxide, and perhaps is also affected by (*c*) the rate of hydrolysis of the depside. The depside gives a slight reaction with aluminum chloride, equal to or less than 10% of that given by rutin.

In preliminary experiments to determine the optimum conditions for the assay, a volume of 4 to 6 ml of fluid per assay tube was found optimal. The effect of substrate and of enzyme concentration on the amount of rutin hydrolyzed is presented in Fig. 1. The optimum concentration of rutin for enzyme activity is 40 mg per assay tube and up to 60% of the substrate can be decomposed without the substrate concentration becoming the rate limiting factor. These data also indicate that high concentrations of rutin are inhibitory. Shaking, which provides aeration and keeps the rutin in suspension,

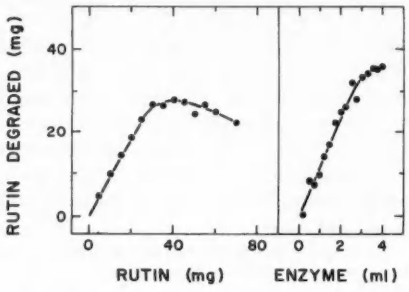

FIG. 1. Effect of rutin and enzyme concentration on the degradation of rutin.

is required to obtain enzymatic activity as rutin was not degraded in quiescent reaction mixtures after 3 hours' incubation at 30° C.

Analytical

Rutin was determined as previously described (15), ammonia by nesslerization (6), and rutinose by anthrone after absorption of flavonoids on magnesol (8, 14). Mycelial weights were determined by collecting the mycelium on a Millipore filter pad (0.45 mμ pore size) and drying to constant weight at 25° C. Enzymatic activity and mycelial weights are reported as the mean and standard error of three replicates.

Experimental

Effect of pH on Activity and Stability of Enzyme System

The optimum pH for degradation of rutin by the extracellular enzyme system is between pH 5.5 and 6.5 (Fig. 2). This is less acidic than the optimal pH of an anthocyanase system, which was also capable of attacking rutin (3, 4, 5).

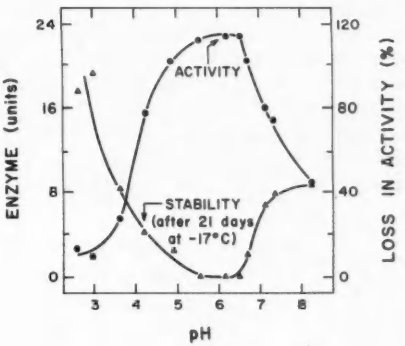

FIG. 2. Effect of pH on activity and on stability of the enzyme system as shown by the percentage loss in activity after storage.

The effect of pH on the stability of the enzyme system during storage was determined by mixing equal volumes of filtrate and citrate–phosphate buffer at the desired pH values, storing the preparations at −17° C, and assaying periodically for activity. Since the enzyme system is quite stable at this temperature when buffered between pH 5.5 and 6.5 (Fig. 2), samples could be taken, the pH adjusted, and stored at −17° C for short periods of time before analysis.

Production in Shaken Erlenmeyer Flasks

Figure 3 shows the relationship between the utilization of rutin, extracellular accumulation of enzyme, growth, and pH. Maximum yield of the enzyme system was attained at 72 hours, after growth and complete utilization of the substrate had occurred. Samples were therefore routinely taken after 72 hours' incubation.

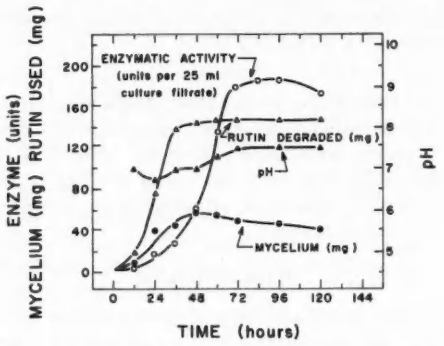

FIG. 3. Production of the enzyme system in shaken Erlenmeyer flasks (25 ml of medium in 250-ml Erlenmeyer flasks).

Effect of Temperature

The influence of temperature on production is presented in Fig. 4. While the amount of enzyme released is independent of the temperatures tested, production is much faster at 35 and 30° C than at 25° C. The rapid decrease in activity of the culture filtrates after the maximum was reached contrasts sharply with the results presented in Fig. 3 where three times the concentration of substrate was used and the enzymatic activity of the filtrates was relatively constant. This suggests that the higher concentration of rutin either stabilized the extracellular enzymes or prolonged production, thus compensating for destruction.

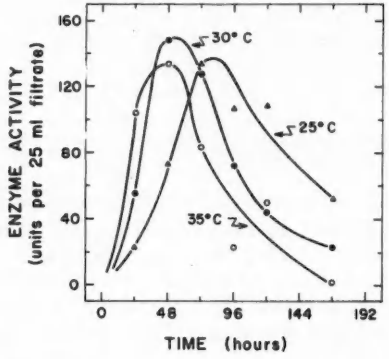

FIG. 4. Influence of temperature on production of the enzyme system.

Effect of Aeration

The degree of aeration, as affected by the volume of medium per flask, is an important factor in obtaining high yields of the enzyme system and complete utilization of the available substrate (Table I). An optimum level of aeration exists since increasing the volume of medium in the 250-ml Erlen-

meyer flasks from 25 ml to 50 ml resulted in an increase in the amount of the ensyme system in the filtrate, but increasing the volume to 100 ml resulted in a decrease. The concentration of rutin is also a factor since increasing the concentration of rutin three times resulted in an increase of approximately 1.5 times the amount of the extracellular ensyme in the medium (Table I).

TABLE I

Effect of aeration on production of the enzyme system and utilization of rutin

Volume per flask	Rutin, %	Enzyme, units per 25 ml		Rutin used, %	
		Shaken	Quiescent	Shaken	Quiescent
25	0.2	213.6 ± 7.55	155.5 ± 5.81	99.0	80.0
	0.6	342.3 ± 4.94	255.5 ± 22.41	97.5	60.5
50	0.2	511.8 ± 0	149.8 ± 26.6	98.9	61.5
	0.6	857.3 ± 6.73	208.0 ± 18.4	99.2	51.2
100	0.2	160.4 ± 56.5	36.5 ± 36.5	98.9	18.0
	0.6	124.0 ± 42.7	120.0 ± 20.0	98.7	—

NOTE: Medium consisted of rutin, 0.15% $(NH_4)_2HPO_4$, 0.01% KH_2PO_4, salts, and vitamins.

Aeration also affects the excretion of the enzyme system into the culture medium (Fig. 5). A volume of 100 ml of medium per 250-ml Erlenmeyer flask appears optimal for synthesis but a volume of 50 ml per flask is optimal for excretion. The decrease in pH with increasing volumes of medium per flask may be attributed to the accumulation of acidic products resulting from depressed oxidation by the mold.

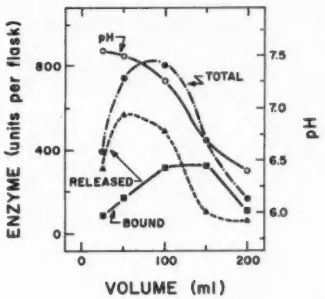

FIG. 5. Effect of volume of medium in the 250-ml Erlenmeyer flasks on production and distribution of the enzyme system.

Development of a Synthetic Medium

Substrate Concentration

That the concentration of rutin has an effect on the extracellular production of the enzyme system was indicated from the data presented in Table I. This observation is confirmed in the results presented in Fig. 6 where a concentration of 0.4% rutin is shown to be required for maximum production.

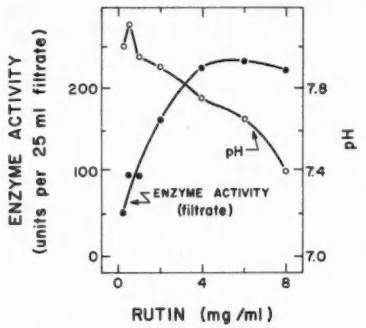

FIG. 6. Effect of concentration of rutin on production of the extracellular enzyme system.

Comparison of Nitrogen Sources

The only organic sources of nitrogen studied that were not suitable were asparagine and urea (Table II). Of the inorganic sources of nitrogen, only ammonium sulphate resulted in a high level of excretion. The observation that the pH of cultures grown on organic nitrogen sources is around 8.0 whereas of those grown on inorganic nitrogen sources is about 6.5 indicates that the mold is using the organic compounds both as sources of nitrogen and carbon.

TABLE II

Effect of the source of nitrogen on production of the enzyme system

Source	Concentration, mg of N/flask	pH	Enzyme activity, units per 25 ml filtrate
Asparagine	212	7.7	46.9 ± 5.7
Casein*	158	8.3	138.1 ± 4.9
N-Z amine†	128	8.6	138.6 ± 2.7
Peptone*	162	8.6	115.1 ± 17.7
Phytone‡	80	8.6	111.1 ± 10.4
Tryptone†	131	8.6	153.8 ± 12.6
Urea§	93	8.3	47.4 ± 1.1
Yeast extract*	94	8.6	161.4 ± 17.5
$(NH_4)_2SO_4$	42	6.4	210.6 ± 6.8
NH_4Cl	52	6.2	77.6 ± 8.9
NH_4NO_3	70	6.4	50.8 ± 8.5
KNO_3	55	6.6	67.4 ± 13.1

*Difco.
†Sheffield.
‡Baltimore Biological Laboratory.
§Sterilized by filtration.
NOTE: Basal medium contained 0.2% rutin, 0.15% K_2HPO_4, 0.01% KH_2PO_4, vitamins, salts, and a source of nitrogen as listed above.

Phosphate Concentration

The effect of phosphate on production of the enzyme is presented in Fig. 7. Production is dependent upon the presence of phosphate, maximum production occurring at a concentration of 0.03 *M* phosphate. The failure to obtain much enzyme at levels of phosphate below 0.015 *M* may be attributed to

inadequate buffering since reversing the ratio of dibasic to monobasic phosphate while maintaining the level of phosphate and of ammonium ion at 0.03 *M* results in pH values during growth of 3.5 and lack of enzyme in the filtrates.

The data in Table III substantiate that phosphate is required for production as the total amount of enzyme synthesized with $CaCO_3$ as the buffer was increased by the addition of phosphate. These data also indicate that phosphate is required for excretion as the addition of phosphate to the medium results in a larger proportion of the enzyme system in the filtrate. However, the use of $CaCO_3$ in the medium results in a smaller proportion of the enzyme system being excreted.

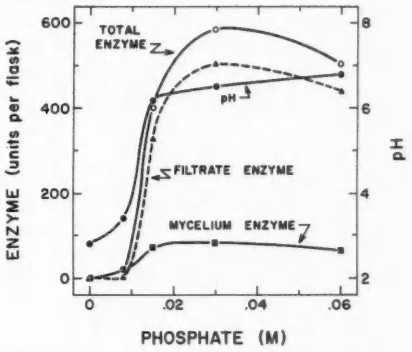

FIG. 7. Effect of concentration of phosphate on production and distribution of the enzyme system in the filtrate and mycelium. (Medium contained 0.4% rutin, 0.8% $(NH_4)_2SO_4$, and salts and phosphate as given.)

TABLE III

Effect of phosphate and calcium carbonate on excretion of the enzyme system

Phosphate, m*M*	$CaCO_3$, m*M*	pH of filtrate	Enzyme, units			% excreted
			Filtrate	Mycelium	Total	
0	0	3.0	23.6 ± 1.1	4.0 ± 3.9	27.6	86
	5	7.2	96.3 ± 11.7	132.5 ± 9.2	228.8	42
	20	7.9	89.3 ± 1.0	202.1 ± 35.9	291.4	31
	80	7.4	68.0 ± 13.2	200.1 ± 2.0	268.1	25
	320	7.4	15.5 ± 9.1	37.2 ± 1.3	52.7	29
1.96	0	3.2	24.8 ± 5.1	0	24.8	100
	5	7.0	198.3 ± 10.9	128.9 ± 18.3	327.2	61
	20	7.7	158.3 ± 6.1	201.4 ± 7.3	359.7	44
	80	7.8	117.4 ± 0.9	241.9 ± 1.6	359.3	33
	320	7.9	14.1 ± 14.1	—*	—	—
15.0	0	6.2	359.3 ± 20.4	74.4 ± 5.8	433.7	83
	5	6.8	231.0 ± 19.3	124.5 ± 16.9	355.5	65
	20	7.3	201.5 ± 5.8	155.0 ± 2.3	357.0	56
	80	7.9	22.6 ± 3.6	—*	—	—
	320	8.1	12.1 ± 4.6	—*	—	—

NOTE: Basal medium consisted of 0.4% rutin, 0.8% $(NH_4)_2SO_4$, and salts.
*Growth scant.

TABLE IV

Effect of vitamins and minor salts on production of rutin degrading system

Medium	Amount per flask	pH of filtrates	Mycelium, mg*	Enzymes, units per flask		
				Filtrate	Mycelium	Total
Basal	—	5.4	8.5	46.4 ± 5.0	3.5 ± 1.7	49.9
" + vitamins	†	5.7	9.7	77.3 ± 13.3	14.7 ± 3.6	92.0
" + salts	†	6.2	27.4	379.1 ± 9.3	31.2 ± 2.0	410.3
" + vitamins + salts	†	6.3	27.3	275.5 ± 40.9	43.1 ± 0.9	318.6
Basal + salts	†	5.5	35.1	198.8 ± 38.5	43.1 ± 14.5	241.9
" + $MgSO_4.7H_2O$	1 mg	4.7	—	13.8‡	—	—
	10 mg	5.3	36.4	73.9 ± 29.2	172.7 ± 20.5	246.6
" + $MgCl_2.6H_2O$	10 mg	5.7	37.3	108.3 ± 14.8	155.9 ± 5.4	264.2
" + $CaCl_2.2H_2O$	10 mg	5.4	8.4	31.0 ± 9.0	—	—
" + $CaSO_4.2H_2O$	10 mg	5.8	7.6	72.9 ± 10.3	22.4 ± 10.5	95.3
" + $ZnSO_4.7H_2O$	1 mg	4.8	10.1	30.7 ± 9.8	—	—
" + $FeSO_4.7H_2O$	1 mg	4.6	8.9	26.4 ± 3.0	—	—
" + $CuSO_4.5H_2O$	1 mg	6.6	—	15.7 ± 1.7	—	—

Basal medium consisted of 0.4% rutin, 0.3% $(NH_4)_2HPO_4$, 0.1% KH_2PO_4, and 0.8% $(NH_4)_2SO_4$.
*Standard error = ± 0.8.
†Concentration of vitamins and salts as given in Methods.
‡Single determination.

Effect of Vitamins and Salts

The addition of vitamins to the basal medium did stimulate the production of the enzyme system in the absence of the minor salts, but depressed synthesis in the presence of minor salts (Table IV). The mixture of vitamins was therefore omitted from the medium. The addition of salts to the basal medium resulted in a marked increase in yield.

A second experiment revealed that increasing the concentration of the salt mixture 10-fold affected neither production of enzyme nor formation of mycelium. Of the elements present in the salt mixture, only magnesium as the chloride or as the sulphate was effective in equalling the effect of the salt mixture. Concentrations of magnesium chloride from 1 to 50 mg per flask gave similar yields. A concentration of 25 mg per flask (0.1%) was selected as adequate. Repeated transfer of *A. flavus* on a salt-deficient medium affected the organism so that the final pH of the medium was considerably lower (pH 3.5–3.8) than that of cultures transferred on media containing minor salts (pH 6.3–6.5).

Sulphate is required for the production of the rutin-degrading enzyme system (Table V). This requirement explains the failure to get high yields in the filtrates when the organism was grown on nitrogen sources such as ammonium chloride. In addition to nutritional requirements, sulphate may be affecting the excretion of the enzyme system as sulphate has been shown to stimulate the release of bound streptomycin from the mycelium of streptomycin-producing actinomycetes (7). A concentration of 0.8% ammonium sulphate proved optimal.

TABLE V

Effect of sulphate on the amount of the enzyme system in filtrates

Nitrogen source	K_2SO_4	pH of filtrate	Enzyme activity, units per flask
KNO_3	0	6.70	9.8
	+	6.70	154.8
NH_4NO_3	0	6.30	7.2
	+	6.30	164.5
NH_4Cl	0	6.40	59.4
	+	6.35	156.6
$(NH_4)_2SO_4$	0	6.50	144.5

NOTE: Basal medium consisted of 0.2% rutin, 0.15% K_2HPO_4, 0.03% KH_2PO_4, salts, and 6 mmoles of nitrogen per liter. Potassium sulphate was added, where indicated, at a concentration of 3 mmoles per liter.

Production in Fermentors of Two Components of the Enzyme System

On the basis of the results presented, culture filtrates of high activity can be obtained with a medium consisting of 0.4% rutin, 0.3% $(NH_4)_2HPO_4$, 0.1% KH_2PO_4, 0.1% $MgCl_2.6H_2O$, and 0.8% $(NH_4)_2SO_4$. Since the two main components of the enzyme system are the glycosidase that hydrolyzes the quercetin–rutinose bond and the enzyme(s) that cleave the heterocyclic ring of quercetin (quercetinase), the production of these two enzymes in a 5-gallon fermentor was followed.

The fermentor consisted of a stainless steel cylindrical tank, 18.5 in. high by 11.5 in. in diameter (inside measurements), similar in design to the 5-liter

stainless steel fermentors previously described (9). The vane-disc impeller had a diameter of 6.5 in. and was situated just above a single nozzle (1/16 in.) aerator. The fermentor was equipped with four 1-in. baffles and an air filter (glass wool).

The medium (18 liters) was prepared by sterilizing the ammonium and potassium phosphates and ammonium sulphate in 15 liters of water in the fermentor in a large autoclave. The rutin and magnesium chloride were sterilized together in 3 liters of water in an aspirator bottle equipped with a hose and nozzle to facilitate aseptic transfer to the fermentor. The inoculum was a 48-hour-old culture of *A. flavus* prepared by inoculating, with a heavy suspension of spores, two Erlenmeyer flasks (500-ml capacity) containing 90 ml of medium and incubating them at 30° C on a rotary shaker. The fermentor was placed in a water bath at 30° C, aerated at the rate of 6 liters of air per minute, and stirred (350 r.p.m.). Samples were withdrawn, aseptically, at intervals and analyzed. Glycosidase activity was measured by Dr. G. Hay, who determined the amount of sugar released from rutin at pH 5.6 under standard conditions. One unit of glycosidase is the amount of enzyme required to release 1 micromole of rutinose per hour. Quercetinase activity was measured in a Beckman spectrophotometer and one unit is defined as the amount of enzyme required to degrade 1 micromole of quercetin per hour as determined by the loss in absorbance at 356 mμ under standard conditions. Both methods will be described in detail in subsequent papers.

The glycosidase and quercetinase are not found in the culture filtrate until most of the substrate has been degraded (Fig. 8), as is commonly observed in production of extracellular enzymes. The glycosidase attains a maximum level shortly after growth ceases, but quercetinase continues to accumulate for another 10 hours. This difference may be attributed to the fact that the

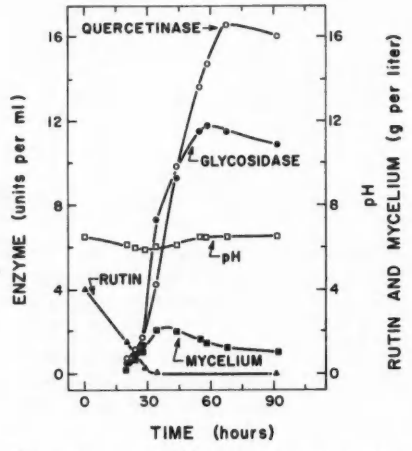

FIG. 8. Production of two components of the enzyme system, glycosidase and quercetinase, in 18 liters of medium in 5-gallon fermentors.

glycosidase is the first enzyme to attack rutin, but more likely is due to the fact that the glycosidase is most stable at pH 5.6 whereas quercetinase is most stable at pH 6.0. Thus, if the glycosidase is desired, the enzyme should be recovered at about 55 to 60 hours, but if quercetinase is wanted the fermentation should be allowed to continue until 65 to 70 hours.

Discussion

Aspergillus flavus, PRL 1805, when grown on a medium containing rutin, phosphate, magnesium, and ammonium sulphate, produces an extracellular enzyme system that degrades rutin. This enzyme system is released into the medium after maximum growth has been attained and all the available substrate utilized. The release could be attributed to autolysis of the mycelium or to increased permeability of the cell walls when the rate of growth decreases and the culture matures. The latter explanation would account for low excretion of the enzyme system when high concentrations of rutin were employed or under conditions of low aeration where the rate of substrate utilization is low.

Regardless of the organic source of nitrogen (urea and asparagine excepted), a considerable amount of the enzyme system was excreted into the medium whereas with the inorganic source of nitrogen only ammonium sulphate resulted in appreciable excretion. This agrees with the work of Feniksova (1), who showed that the amylase of *Aspergillus oryzae* was bound in the mycelium when grown on nitrate but was excreted when grown on ammonium sulphate (in the absence of phosphate). The lower yields obtained on organic nitrogen sources as compared to ammonium sulphate can be explained if one assumes that a high organic nitrogen content in the medium inhibits the release of the mycelial constituents.

Phosphate is also required for excretion of the enzyme system. This anion has been demonstrated to be required for the extracellular production of amylase by *A. oryzae*, to stimulate the production of amylase by *Bacillus subtilis*, and to favor the production of proteinases (1, 2, 13).

The presence of small amounts of $CaCO_3$, enough to maintain a neutral pH when the phosphate level is inadequate for buffering, is beneficial for enzyme production. However, $CaCO_3$ inhibits the release of the enzyme system. As the concentration of $CaCO_3$ is increased, the amount of the enzyme system released decreases and the amount bound increases. The inhibitory effect of $CaCO_3$ probably arises from at least two sources: the calcium ion combining with the phosphate anion forming insoluble calcium phosphates and by the carbonate anion rendering the heavy metals insoluble and unavailable. Simpson similarly reports that high concentrations of $CaCO_3$, but still less than 1%, inhibited the production of pentosanases by both *B. subtilis* and *A. niger* (10, 11).

Acknowledgments

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PREPARATION OF CELL-FREE STAPHYLOCOCCAL PENICILLINASE CONCENTRATE[1]

M. GOLDNER AND R. J. WILSON

Abstract

The preparation of staphylococcal intracellular penicillinase as a cell-free concentrate is described and the optimal conditions for its action on penicillin indicated. Cells were disrupted by sonic vibrations and the debris removed by centrifugation at 17000×*g*. Cell-free concentrates were prepared from the supernatants and were found to have a higher activity per mg nitrogen than the bacterial suspensions from which they were prepared. Centrifugation at 35000 ×*g* resulted in a partial and as yet unaccountable loss of activity. The enzyme was optimally active at pH 6.0–6.5 and 30° C. The possible application of cell-free staphylococcal penicillinase to enzymatic and immunological work is discussed.

Introduction

The *penicillin resistance of many clinically isolated strains of staphylococci* is considered to be due to the penicillinase produced by these organisms (2, 13). The action of penicillinase on penicillin results in the formation of antibiotically inactive penicilloic acid (1). It has been reported that staphylococcal penicillinase is an inducible enzyme (4, 5) and we have found that growth of penicillin-resistant strains in the presence of penicillin results in a higher penicillinase production than does growth without penicillin.

In all of the penicillinase-producing strains of staphylococci examined by us, the enzyme was found to be intracellular. Hitherto, preparations of staphylococcal penicillinase have been in the form of defatted non-viable cells containing the enzyme, prepared according to the method of Harper (6). However, for antigenic use a soluble preparation of the enzyme is desirable and such a preparation would also be helpful in inhibition studies with chemical compounds. The object of the present work was to obtain an active cell-free enzyme preparation by disrupting the cells with sonic vibrations and preparing a concentrate from the sonic lysate after centrifugation. The essence of this work is that the enzyme preparations are cell free. No claim is made that the penicillinase concentrates when dispersed in liquid are actually in the form of what is considered to be 'soluble enzyme'.

Methods

Preparation of Penicillinase

A clinically isolated strain of *Staphylococcus aureus*, Saskatchewan No. 755 (phage type 81),* was used as the source of penicillinase. The sensitivity range of this strain was 4–8 μg sodium benzyl penicillin per ml (tube dilution

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Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario. Presented in part to the annual meeting of the Canadian Society of Microbiologists, Quebec City, June, 1959.

*Obtained from Dr. H. O. Dillenberg, Department of Public Health, Province of Saskatchewan, Regina, Saskatchewan.

method, small inoculum) and it formed significant levels of basal penicillinase as well as increased amounts when grown in the presence of penicillin. The microorganisms were grown in 1-liter amounts of nutrient broth in 3-liter Erlenmeyer flasks for 24 hours at 36–37° C with constant shaking. The inoculum consisted of 10 ml of a heavy suspension in physiological saline of bacteria grown for 12 hours on nutrient agar slopes. Penicillin was added to the culture in order to increase the penicillinase production; 10 units (u.) of potassium benzyl penicillin per ml was added $2\frac{1}{2}$ hours after inoculation, near the beginning of the exponential phase. A second addition of penicillin, at final concentration 1000 u. per ml, was made $5\frac{1}{2}$ hours after inoculation. The staphylococcal cells were collected by centrifugation and washed with distilled water. The cells were suspended in 50 ml of 0.05 *M* phosphate buffer, pH 6.25, containing 10% sucrose (w/v), 0.02 *M* NaCl, and 0.003 *M* $MgSO_4$ (3) at a density of 20–25 mg bacterial dry weight per ml. The suspended cells were subjected to sonic vibrations at 10 kilocycles per second for $1\frac{1}{2}$ hours using a 250-w Raytheon sonic oscillator. The suspension of disrupted cells was centrifuged in the cold at 17000 $\times g$ (30 minutes) and the supernatant concentrated by lyophilization. The lyophilized material was transferred to a dialysis sac with a minimal volume of 0.1 *M* phosphate buffer, pH 7.0, and dialyzed three times against 0.01 *M* phosphate buffer, pH 7.0. The preparation resulting from the lyophilization of the contents of the dialysis sac was termed a penicillinase 'concentrate'. When 0.1 *M* phosphate buffer, pH 7.0, was added to this concentrate, the resulting liquid, which was slightly cloudy in appearance, was clarified after centrifugation at 35000$\times g$. In another instance, a disrupted cell suspension was centrifuged at 35000$\times g$ (60 minutes) and a 'concentrate' prepared from the supernatant. When the phosphate buffer was added to this concentrate, the resulting liquid was slightly cloudy in appearance but centrifugation at 17000 $\times g$ was sufficient to achieve clarification. The slight sediment obtained was found to possess penicillinase activity.

Measurement of Penicillinase Activity and Nitrogen Content

The enzyme activity was determined by the iodometric method, according to the procedures of Perret (9) and Tucker (14). This method is based on the reaction of iodine with penicilloic acid, the product of the enzyme reaction on penicillin. Enzyme activities were expressed as micrograms of penicillin hydrolyzed per minute at pH 6.5 and 30° C. The micro-Kjeldahl method of Ma and Zuazaga (7) was used for the nitrogen assays.

Results

In Table I are shown the yields and activities of three cell-free preparations of staphylococcal penicillinase. Preparations I, II, and III are concentrates prepared from supernatants after centrifugation at 17000$\times g$, 17000$\times g$, and 35000$\times g$, respectively. The yield was approximately 0.4 g of concentrate per liter of culture. The yield of preparation I in terms of mg penicillinase preparation per mg bacterial nitrogen was less than that of preparation II. The yield of preparation III in the same terms was nearly the same as that of preparation II, although the former was prepared from the supernatant obtained after a higher speed of centrifugation. Preparations I and II con-

tained 25 and 37% respectively, of the total nitrogen content of the bacterial suspensions from which they were prepared, while preparation III contained only 12%. The nitrogen content of preparations I and II was three to three and one-half times greater than that of preparation III (Table I).

TABLE I

Yield and activity of *S. aureus* penicillinase preparations*

Preparation	Yield (mg penicillinase preparation/mg bacterial N)	Activity		
		Total activity of preparation /mg bacterial N	Specific activity, per mg preparation dry wt.	Specific activity, per mg preparation nitrogen
		(μg potassium benzyl penicillin hydrolyzed/min)		
I (11.2% N)	2.25	207	92	817
II (9.3% N)	3.94	376	95	1023
III (3.2% N)	3.66	95	26	795

*Concentrates prepared from supernatants after sonic disruption of suspensions of staphylococcal cells (as described under Methods): preparations I and II, 17000 $\times g$ concentrates; preparation III, 35000 $\times g$ concentrate.

The total activities of preparations I and II were three to four times that of preparation III per liter of culture. Per mg of bacterial nitrogen, the total activities of preparations I and II are calculated to be, respectively, 2.2 and 4.0 times that of preparation III. The lower activity of preparation III would appear to be the result of centrifugation at the higher speed. The specific activity of preparation I is the same as that of preparation II on a dry weight basis but is somewhat lower on a nitrogen basis. The specific activity of preparation III is about one-quarter of that of preparations I and II on a dry weight basis but is similar to that of preparation I on a nitrogen basis. The ratio of the total activity of the preparation to the total activity of the bacterial suspension is given in Table II. We see that 40 to 60% of the original activity of the suspension has been recovered in preparations I and II whereas only 8% has been recovered in preparation III. Furthermore, it should be noted from Table II that the ratio of the specific activity of the preparation to the specific activity of the bacterial suspension shows an increase of 50 to 70% for preparations I and II but a decrease of 36% for preparation III on a nitrogen basis.

When the concentrates prepared from the 17000 $\times g$ supernatants (e.g. preparations I and II) were dispersed in 0.1 *M* phosphate buffer, pH 7.0, at low concentrations, a slight turbidity was observed. When one of these

TABLE II

Ratio of activity of *S. aureus* penicillinase preparations to activity of bacterial suspensions

Preparation	Ratio of total activity of preparation: total activity of bacterial suspension	Ratio of specific activity of preparation: specific activity of bacterial suspension (nitrogen basis)
I	0.39	1.53
II	0.62	1.71
III	0.08	0.64

suspensions was centrifuged at 35000×*g*, the activity in the supernatant was approximately 20% of that present in the supernatant of a similar suspension after 17000×*g* centrifugation. Also, in this regard, it was found that when the buffer was added to preparation I giving a 50 mg% concentration, nearly one-third of the activity was unaccounted for after its distribution between sediment and supernatant by 35000×*g* centrifugation. With preparation I at one-half the previous concentration, the percentage of the activity recovered in the supernatant after 35000×*g* centrifugation was less than one-half of the percentage recovered at the higher concentration. Explanation of this loss of activity requires further investigation.

The optimum pH and the optimum temperature for cell-free staphylococcal penicillinase activity were determined after a reaction time of 60 minutes. The preparation used was the supernatant obtained after 35000×*g* centrifugation of preparation I in 0.1 *M* phosphate buffer, pH 7.0, at a 25 mg% concentration, with 0.5% gelatin added to the supernatant for stabilization. From the activity vs. pH curve (Fig. 1), it can be seen that the optimum pH was 6 to 6.5. At the more acid pH of 5, 60% of the maximum activity was present but at pH 8, on the alkaline side, only 33% of the maximum activity was found. Pollock (10) reported a pH optimum of 6 for *Bacillus cereus* 569 γ-penicillinase (cell-bound, unneutralizable by antiserum to extracellular enzyme), with the activity decreasing sharply on either side of the range 5.5 to 6.5. Previously, Manson, Pollock, and Tridgell (8) had found that pH 6 was optimum for *B. cereus* 569 extracellular penicillinase also, but with the activity decreasing gradually on either side of the range 5.5 to 7. From the activity vs. temperature curve (Fig. 2), it can be seen that the optimum temperature was 30° C. This curve was obtained using Perret's method and

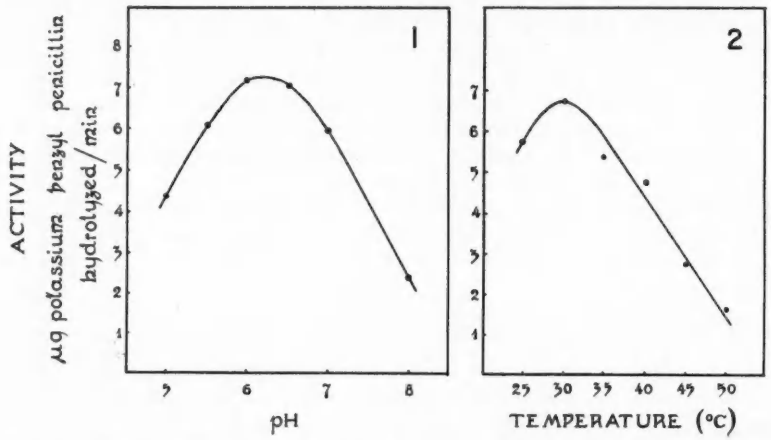

FIG. 1. Effect of pH on the activity of *S. aureus* penicillinase at 30° C (reaction time, 60 minutes).

FIG. 2. Effect of temperature on the activity of *S. aureus* penicillinase at pH 6.5 (reaction time, 60 minutes).

it should be noted that the activity was only 40 to 70% of maximum between 40° and 45° C, which was shown by Perret (9) to be the optimum range for *B. cereus* 569 extracellular penicillinase at pH 6.5. At 35° to 40° C, which was the optimum temperature range obtained by Manson *et al.* (8) for the extracellular penicillinase of *B. cereus* using a manometric method and a pH of 7, Fig. 2 shows the staphylococcal penicillinase to have only 70 to 80% of its maximum activity.

Some reaction rates with various concentrations of the gelatin-stabilized enzyme at pH 6.5 and 30° C were determined. When the velocity constant, *K*, was calculated for each concentration of penicillinase according to the first order reaction formula and plotted against the corresponding enzyme concentration, a direct linear relationship was obtained (Fig. 3). The rate of penicillin hydrolysis thus depends solely on the enzyme concentration, provided the substrate is in excess.

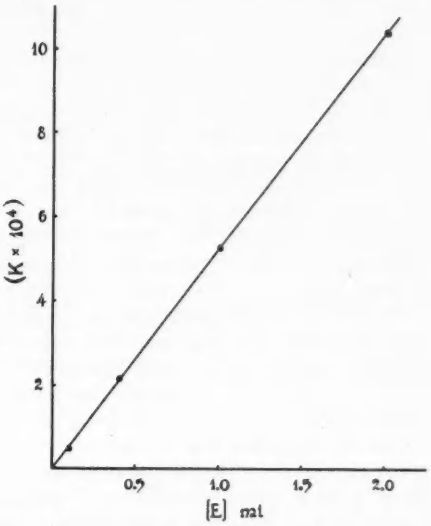

FIG. 3. Relationship of velocity constant to *S. aureus* penicillinase concentration.

Discussion and Conclusions

It has been possible to obtain cell-free concentrates of staphylococcal intracellular penicillinase after sonic disruption of the cells. The enzyme was concentrated by dialysis and lyophilization, yielding approximately 2 to 4 mg of preparation per mg nitrogen of the bacterial source. The yield per mg bacterial nitrogen would depend on the extent of disintegration of the cells although the yield per liter of culture would vary according to the total growth.

When the specific activities of the concentrates were compared on a dry weight basis, it was realized that the concentrate prepared from the supernatant after 17000×*g* was three and one-half times more active than the concentrate prepared from the supernatant after 35000×*g*. The nitrogen content per mg

of the former concentrate being also three to three and one-half times the nitrogen content of the latter concentrate, a possible correlation between activity and content of nitrogen was thus seen.

Under the conditions of cell disruption used in the preparation of staphylococcal penicillinase concentrates described in this report, it was found that centrifugation of the suspension at $35000 \times g$ resulted in a concentrate having approximately one-tenth of the original total activity while centrifugation at $17000 \times g$ resulted in recovery of 40 to 60% of the original total activity. Pollock (10) has used cell disruption as a means of isolating intracellular penicillinase from *B. cereus* 569 and was able to obtain approximately 25 to 50% of the γ-penicillinase in a soluble state. Sheinin (12) found that sonic treatment of membranes isolated from *B. cereus* 569/H cells made it possible to obtain γ-penicillinase in a soluble form (non-sedimentable at $105000 \times g$). Geronimus and Cohen (5) have made use of sonic disruption of staphylococcal cells in connection with their work on inducibility of staphylococcal penicillinase.

The conclusion might be drawn that more extensive disintegration would result in the recovery of more activity at the higher speed of centrifugation. However, it was observed that, compared to the original bacterial suspension, the activity per mg nitrogen was decreased in the $35000 \times g$ concentrate by approximately one-third. In contrast to this decrease, the activity of the $17000 \times g$ concentrate had increased by 50 to 70% per mg nitrogen, which seemed to indicate that there was some other factor besides disintegration to consider. This was further supported by the observation that, after the centrifugation at $35000 \times g$ of a dilute preparation (in buffer) of a $17000 \times g$ concentrate, a significant amount of the original activity was not recovered. It was possible that this loss of activity resulted from instability of the enzyme at low concentrations. To ensure enzyme stability, gelatin was incorporated in the preparations used in the determinations illustrated in Figs. 1, 2, and 3. The addition of gelatin for this purpose to preparations of *B. cereus* penicillinase has been advocated by Perret (9) and by Pollock, Torriani, and Tridgell (11). The effect of lyophilization on the concentrates prepared from the supernatants at the two speeds of centrifugation was not studied.

The optimum pH of a preparation of cell-free *S. aureus* penicillinase was revealed to be similar to that of *B. cereus* penicillinases, both cell-bound and extracellular. There was also a close similarity in the activity vs. pH curves of the *S. aureus* penicillinase and the *B. cereus* γ-penicillinase. The optimum temperature of the staphylococcal penicillinase preparation differed from that of *B. cereus* extracellular penicillinase by at least 5° C.

Staphylococcus aureus cells producing penicillinase have been sonically disrupted and the enzyme prepared in the form of cell-free concentrates. An optimum pH of 6 to 6.5 and an optimum temperature of 30° C were observed. Cell-free concentrates of isolated staphylococcal penicillinase could be used for the subsequent preparation of purified enzyme. The purified penicillinase would be helpful in inhibition and antigenic studies in connection with staphylococcal resistance to penicillin. Further investigation into the matters of the enzyme stability and the localization of penicillinase in the staphylococcal cell (12) is contemplated.

Acknowledgments

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SPECIFIC DEGRADATION OF CRYPTOCOCCUS NEOFORMANS 3723 CAPSULAR POLYSACCHARIDE BY A MICROBIAL ENZYME

I. ISOLATION, PARTIAL PURIFICATION, AND PROPERTIES OF THE ENZYME[1]

HANS H. GADEBUSCH AND JOHN D. JOHNSON

Abstract

A partially purified intracellular enzyme from a species of *Alcaligenes* is described which specifically initiates the degradation of the heteropolysaccharide of *Cryptococcus neoformans*, isolate 3723. The enzyme is active in the presence of serum and can be inactivated by heating at 45° C for 10 minutes. The kinetics of the enzyme reaction are similar to those of other enzymes. Recovery and identification of the four known monosaccharides from enzymatic hydrolyzates suggest the presence of a number of other enzymes in these preparations.

Heteropolysaccharases have been only rarely described and appear to be very restricted in occurrence. Most often they are isolated after soil enrichment with the particular polysaccharide under investigation. In this manner type-specific pneumococcal polysaccharases (1, 2) and certain group-specific streptococcal polysaccharases (3) have been isolated from soil organisms.

Recently we have isolated from soil a bacterium (*Alcaligenes* sp. S-3723) which not only degrades the purified capsular heteropolysaccharide of *Cryptococcus neoformans*, isolate 3723, but also decapsulates the parent fungus (4). This paper will discuss the isolation and properties of the enzyme(s) responsible for this action.

Materials and Methods

Purified capsular polysaccharides from *Cryptococcus neoformans*, isolates 3723, BRI, DU, and TRE and their respective antisera were prepared by the methods of Gadebusch (5, 6). *Diplococcus pneumoniae*, types I, II, III, and *Klebsiella pneumoniae*, type B, and their antisera were prepared according to Heidelberger (7, 8) and Avery (9). All sera were absorbed to remove serological cross reactions (6). Anti-BRI was absorbed with DU cells and anti-TRE with DU and 3723 cells. The antibody contents of the sera were sufficiently high to permit reproducible results in the tests employed. Protein was determined by the biuret method of Robinson and Hogden (10). D(+)-galactose, D(+)-mannose, D(+)-xylose, and glucuronic acid were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. All other chemicals used were commercial samples of reagent grade purity.

Experimental and Results

Measurement of Enzyme Activity

The standard assay system (SAS) contained 10 mg/ml of *C. neoformans* 3723 polysaccharide in 0.9 ml of 0.05 *M* NaCl, 0.067 *M* phosphate buffer

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Contribution from the General Medical Research Unit, Veterans Administration Hospital, Ann Arbor, Michigan. This work was presented in part at the 60th General Meeting of the Society of American Bacteriologists, Philadelphia, Pennsylvania, May 5, 1960.

(pH 6.8), and 0.1 ml of properly diluted enzyme solution. Incubation was for 6 hours at 28° C. The reaction was stopped by heating the solution for 15 minutes at 45° C and the polysaccharide remaining was adsorbed on Amberlite* XE-67 anion exchange resin. Standardized serial dilutions of the antigen-sensitized resin suspension were mixed with a constant optimal amount of *C. neoformans* 3723 antiserum for the performance of the "particle agglutination test" (11) (PAT). The minimum quantity of enzyme required to degrade completely (no titer in the PAT) 0.1 mg of the polysaccharide under the above conditions was designated as 1 unit.

Isolation of the Enzyme

Twelve batches of the mineral medium (12) containing 10 mg/ml of *C. neoformans* 3723 specific polysaccharide were seeded with heavy inocula of *Alcaligenes* sp. S-3723 and incubated for 24 hours at 28 °C with occasional shaking. Cells and culture supernatant fluid were separated by centrifugation in the cold at 14,000 × *g* for 1 hour and frozen at −27° C. On the following day the cell cake was repeatedly thawed and frozen 4 times in a range of from +10° C to −27° C. A sample of the thawed material was centrifuged in the cold to remove cell debris and compared for enzyme activity with the original supernatant fluid in the SAS. Only the cell extract proved to be active.

The remaining cell mass was therefore shaken in a minimal amount of ice-cold 0.067 *M* phosphate buffer (pH 6.8) at 5° C to produce a uniform suspension which was then centrifuged at 18,000 × *g* for 1 hour. The slightly cloudy, colorless supernatant fluid was decanted, dialyzed against several changes of the same buffer, lyophilized, and stored at 5° C in sealed vials.

Concentration and Partial Purification of the Enzyme

The lyophilized enzyme preparation was reconstituted in 1/10th its original volume of 0.067 *M* phosphate buffer (pH 6.8) and fractionated with neutral ammonium sulphate† at 25° C. Fractions were collected from 0 to 1, 1 to 5, 5 to 10, and 10 to 25% saturation. These fractions were dissolved in 1/10th the original volume of 0.067 *M* phosphate buffer (pH 6.8), dialyzed for 12 hours against repeated changes of glass-distilled water, and lyophilized. A typical analysis is shown in Table I.

TABLE I

Comparative activity of *Alcaligenes* sp. S-3723 enzyme fractions

	Total activity (units)	Specific activity (units/mg protein)
Supernatant I	Inactive	Inactive
Cell extract	7500	0.86
Supernatant II	7300	0.87
Crude enzyme	4210	3.00
$(NH_4)_2SO_4$ fraction after dialysis		
(I) 0–1% sat.	1080	0.92
(II) 1–5% sat.	2150	11.20
(III) 5–10% sat.	1960	11.02
(IV) 10–25% sat.	960	1.01

*Trademark, Rohm and Haas Company, Philadelphia, Pa.
†Saturated $(NH_4)_2SO_4$ at 25° C (4.1 *M*).

Crude enzyme solutions were generally stable for 2–3 weeks in the frozen state. Lyophilized, unfractionated enzyme preparations stored in sealed vials lost little activity on standing in the cold for 3 months. After ammonium sulphate fractionation, solutions were stable for less than 1 week.

TABLE II

Specificity of enzyme fraction II*

Polysaccharide	Precipitin reaction
D. pneumoniae type I	+++
D. pneumoniae type II	+++
D. pneumoniae type III	+++
K. pneumoniae type B	+++
C. neoformans 3723	–
C. neoformans BRI	+++
C. neoformans DU	+++
C. neoformans TRE	+++
Gum acacia	+++

*For procedure see text.

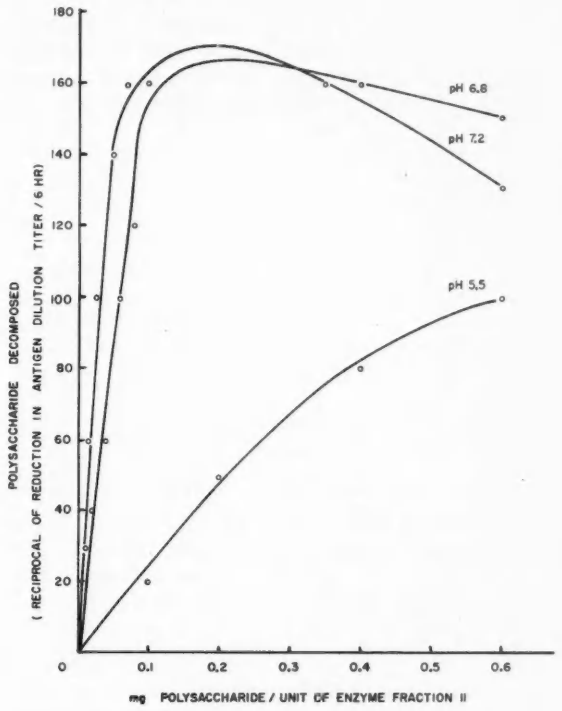

FIG. 1. Effect of substrate concentration on polysaccharide decomposition by enzyme fraction II.

Properties of the Enzyme

The standard assay system using "crude" enzyme (Table I) was employed to determine (*a*) effect of heat, (*b*) effect of aerobic (exposed to the air) and anaerobic (10% CO_2 atmosphere) conditions, and (*c*) effect of animal serum on the activity of the enzyme. It was found that enzyme activity was maintained equally well under aerobic and anaerobic conditions and in the presence of 10% (v/v) human, rabbit, or mouse serum. On the other hand, heating at 45° C for 10 minutes completely inactivated the enzyme.

The specificity of the enzyme was determined by exposing polysaccharides from *D. pneumoniae*, types I, II, and III; *K. pneumoniae*, type B; *C. neoformans*, isolates 3723, BRI, DU, and TRE; and gum acacia to 10 units of enzyme fraction II in the SAS. The mixtures were incubated at 28° C for 7 days and the presence of polysaccharide determined by the qualitative precipitin reaction employing homologous absorbed antiserum. The results (Table II) indicated that the enzyme extracted from *Alcaligenes* sp. S-3723 is indeed as specific in its action as the whole organism (4).

Kinetics of the Enzyme Reaction

The influence of substrate concentration on enzyme activity (fraction II and III) was determined after 6 hours incubation at 28° C. Since fractions II and III gave nearly identical reaction rates, only the data for fraction II are shown in Fig. 1. In polysaccharide solutions below 10 mg/ml (pH 6.8) the reaction obeyed first-order kinetics and changed to zero order at higher

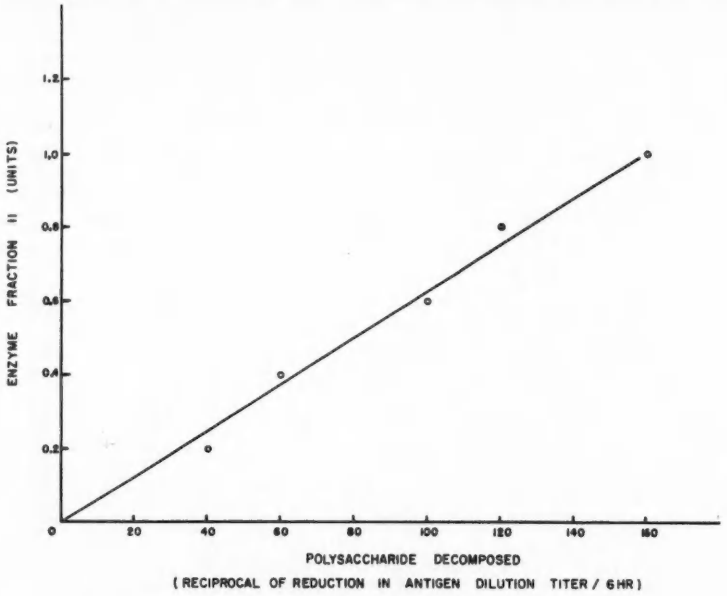

FIG. 2. Reaction velocity as a function of enzyme concentration.

substrate concentrations. When the concentration of polysaccharide was quadrupled in the system, enzyme activity decreased especially at pH values above the optimum for this reaction.

The influence of enzyme concentration upon reaction velocity was determined in the SAS by providing a slight excess of polysaccharide and measuring the polysaccharide decomposed in 6 hours as a function of the reduction in antigen dilution titer (Fig. 2). The reaction velocity under these conditions was directly proportional to the enzyme concentration.

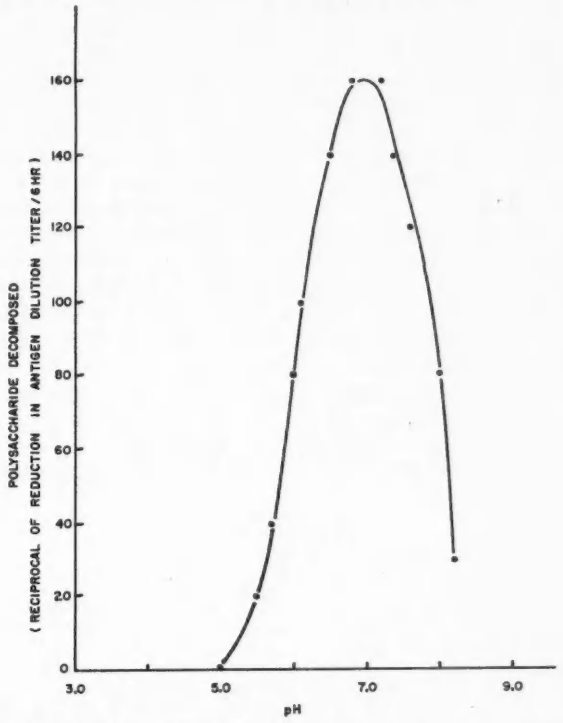

FIG. 3. Effect of pH on polysaccharide decomposition by enzyme fraction II.

The influence of pH upon enzyme activity was determined after incubation for 6 hours at 28° C in Sorenson phosphate buffer containing 0.1 mg polysaccharide per unit of enzyme fraction II (Fig. 3). The pH range of optimum activity in this system was pH 6.8 – 7.2, although the data from Fig. 1 suggest a somewhat narrower range.

Products of Enzymatic Action

In order to secure information about the products of enzymatic action, 0.6 mg *C. neoformans* 3723 polysaccharide was incubated separately in the presence of sufficient enzyme fraction II and III (pH 6.8, 28° C) to degrade all

of the material in 6 hours (no titer in PAT). A sample from each hydrolyzate was removed and the remainder reincubated for an additional 2 hours. The enzyme in all hydrolyzates was inactivated by heating for 15 minutes at 45° C. For comparison purposes a similar amount of polysaccharide was subjected to acid hydrolysis (3 *N* H_2SO_4) for 6 hours at 100° C in sealed ampoules. After hydrolysis the sample was neutralized with 3 *N* NaOH and diluted fivefold with glass-distilled water.

All hydrolyzates were subsequently subjected to descending chromatography in a *n*-butanol–pyridine–water (10:3:3) system (13) with development as follows: 20.5 hours, dried; 17 hours, dried; 14 hours, dried. Separated components were detected by the methods of Durso and Paulson (14).

The comparative data (Fig. 4) demonstrated the formation of four hydrolytic products which were tentatively identified as galactose, mannose, xylose, and glucuronic acid. Since these compounds had previously been identified from paper chromatograms of acid-hydrolyzed polysaccharide (15, 16) confirmatory evidence was limited to the specific spectrochemical procedure of Dische (17) (glucuronic acid) and cochromatography (galactose, mannose, xylose). The unknown sugars produced by enzymatic hydrolysis were eluted from the paper with triple-distilled water (pH 7.0) and a portion tested for total reducing sugar (18). The remainder was mixed with an equal quantity (based on total reducing sugar) of the known carbohydrate and chromatographed in the previously described manner. In every instance, only one spot was detected.

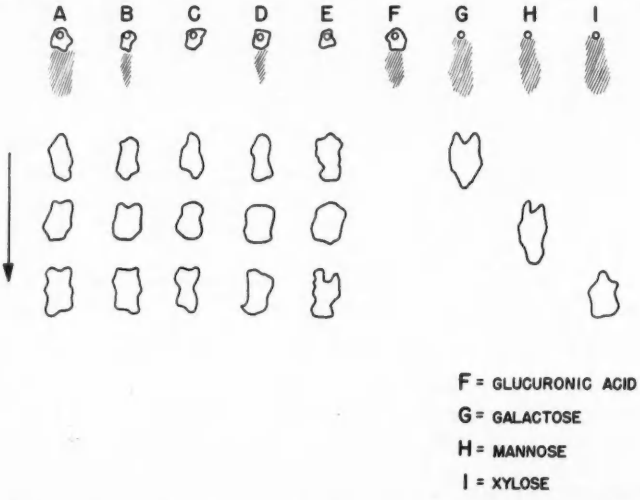

FIG. 4. Composite tracing of paper chromatograms of *C. neoformans* 3723 purified polysaccharide after acid and enzyme hydrolysis. A. Polysaccharide; 6-hour acid hydrolysis. B. Polysaccharide; 6-hour enzyme fraction II hydrolysis. C. Polysaccharide; 8-hour enzyme fraction II hydrolysis. D. Polysaccharide; 6-hour enzyme fraction III hydrolysis. E. Polysaccharide; 8-hour enzyme fraction III hydrolysis. F–I. Known sugars; 6-hour acid hydrolysis.

Discussion

The specific intracellular principle described in these studies appears to be a hydrolytic, adaptive enzyme. Under suitable conditions it is capable of initiating the splitting of the purified capsular polysaccharide of *C. neoformans* 3723, but does not attack even closely related heteropolysaccharides. Since cryptococcal polysaccharides in general show striking similarities with respect to their chemical composition, infrared spectra, and ultraviolet absorbency (4, 19), the specificity exhibited by this enzyme is even more remarkable.

The enzyme may be quantitatively precipitated with ammonium sulphate between the limits of 1 and 10% saturation. By this procedure a 10-fold purification is accomplished. Both active fractions appear to be similar, if not identical, with respect to the rate of the enzyme reaction.

The kinetics of the enzyme reaction are not unlike those of any other enzyme. At low concentrations of substrate the reaction follows first-order kinetics, changes to zero order as the concentration is increased, and at very high substrate levels the reaction proceeds slowly.

The recovery and subsequent identification of the four known monosaccharides from the enzymatic hydrolyzate (enzyme fraction II) suggest the presence of other enzymes in this preparation. This possibility is currently under investigation.

Since enzyme fraction II does not degrade the component monosaccharide sugars (20) and these all possess reducing properties, it is possible to quantitate the rate of reaction of all the enzymes in fraction II by measuring reducing sugar formed. However, it should be pointed out that the rate of reducing sugar formation will lag somewhat in comparison with polysaccharide decomposition as measured by the particle agglutination test.

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OXIDATION OF GLUCONATE, 2-DEOXY-D-GLUCOSE, AND LACTIC ACID BY AN ENZYME PREPARATION FROM AEROBACTER AEROGENES[1]

E. R. BLAKLEY AND O. CIFERRI[2]

Abstract

A particulate preparation from extracts of *Aerobacter aerogenes* oxidized 2-deoxy-D-glucose to 2-deoxy-D-gluconate, 6-deoxy-6-fluoro-D-glucose to 6-deoxy-6-fluoro-2-keto-D-gluconate, and D- and L-lactate to pyruvate.

The oxidations of D-gluconate and D- and L-lactate were found to be different with regard to pH of optimum activity, and to various inhibitory compounds. On the basis of these and other observations, it is suggested that the three substrates were oxidized by different enzyme systems. Other results suggest that the oxidation reactions were catalyzed by flavoprotein enzymes.

Introduction

Dalby and Blackwood (7) described the preparation of a particulate fraction from extracts of *Aerobacter aerogenes* that catalyzed the uptake of 1.0 and 0.5 moles of oxygen per mole of D-glucose and D-gluconate, respectively. They showed that two enzymes were involved, one converting D-glucose to D-gluconate, and a second converting D-gluconate to a ketogluconate. From a study on the oxidation of several substrates, they concluded that the oxidation by the particulate fraction was specific for the D-glucose configuration at carbon-2 and -4. In the present work, however, we found that 2-deoxy-D-glucose was readily oxidized by the particulate fraction. The preparation was also tested for the ability to oxidize substrates not previously tested (7). Both D- and L-lactate were found to be oxidized. The results indicate that the particulate preparation contains several oxidizing enzymes and that the structure of D-glucose at carbon-2 and -6 may be changed without destroying its activity as a substrate.

Materials and Methods

2-Deoxy-D-glucose was obtained from the Sigma Chemical Company or synthesized by the method described by Overend *et al.* (31). The chemically prepared sample, after storage for 2 to 3 years, and the commercial sample were found by paper chromatography to contain an impurity having a lower R_f value than 2-deoxy-D-glucose in butanol–pyridine–water (50:20:18) or ethanol–methanol–water (45:45:10). The impurity reacted with alkaline silver nitrate (36) but not with aniline phthalate (33), and could not be removed from the 2-deoxy-D-glucose by ion exchange resins, charcoal, or recrystallization. A sample of pure 2-deoxy-D-glucose was prepared from 2-deoxy-D-

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glucose dibenzyl mercaptal as described by Overend *et al.* (31). 2-Deoxy-D-gluconate was prepared by bromine oxidation of 2-deoxy-D-glucose according to the method used by Overend and Wiggins (32) for the preparation of D-arabonate. The product was isolated as the barium salt and converted to the free acid by treatment with Amberlite IR-120 (H) resin. 2-Deoxy-D-gluconolactone was prepared as described by Hughes *et al.* (19). 6-Deoxy-6-fluoro-D-glucose was prepared by a modification (3) of the method described by Helferich (15, 16). 6-Deoxy-D-glucose and D-glucosaccharic acid were obtained from Dr. P. A. J. Gorin and Dr. G. Talbot, respectively, of this laboratory. D(−)- and L(+)-lactic acid, purchased from the Mann Chemical Company as calcium salts, were converted to their lithium salts after removal of calcium by ion exchange resin by neutralizing with LiOH and crystallized by the addition of ethanol (2). By optical rotation measurements it was found that the L(+)-lactate contained approximately 20% of the D(−)-lactate. Catalase was obtained from Worthington Biochemical Corporation and lactic acid dehydrogenase from Nutritional Biochemical Corporation.

Preparation of Particulate Material

The organism was grown in the medium reported by Dalby and Blackwood (7) except that the concentration of $MgSO_4$ and KCl was reduced to 1/10th of that reported. The particulate fraction was prepared by the procedure described by Dalby and Blackwood, and was used without lyophilization unless otherwise noted. The preparation could be stored at −10° C for 2 weeks without appreciable loss in activity.

Experimental Procedures

The oxidation of the substrates by the particulate preparation was determined by measuring the consumption of oxygen by conventional Warburg techniques. Unless otherwise noted, each flask contained 20 μmoles substrate in the side arm. The main compartment contained 1 ml of the particulate material suspended in water, 30 μmoles $MgCl_2$, 225 μmoles phosphate, and water to 3.0 ml. The center well contained 0.2 ml of 20% KOH absorbed on a folded filter paper. For most of the experiments the particulate material was resuspended in water to a volume the same as that before ultracentrifugation. This corresponds to approximately 8 mg lyophilized powder or 0.8 mg protein N per milliliter.

Lactic acid racemase activity was determined in the following way. Fifty micromoles of lactate was incubated for 2 hours in an evacuated Thunberg tube with 2 ml particulate material suspended in water, 60 μmoles $MgCl_2$ and 500 μmoles phosphate, pH 6.5, in a total volume of 4 ml. The reaction mixture was then heated 15 minutes in a boiling water bath to inactivate the enzyme and 0.5 ml 10 *N* H_2SO_4 added.

The lactic acid was removed by continuous extraction with ether for 12 hours, diluted with water, and titrated with NaOH to pH 7, then evaporated to dryness. The recovery of lactic acid was quantitative. The dry sodium lactate was dissolved in 2.0 ml water and 0.2 ml assayed for the presence of L-lactic acid with lactic acid dehydrogenase as described by Hohorst (17). Pyruvate was determined by the method of Friedemann (13).

Chromatography

Examination of the products of the reactions was done by ascending paper chromatography with Whatman No. 54 paper using solvent systems as indicated in the text. The spots were located with alkaline silver nitrate (31), aniline phthalate (33), *o*-phenylenediamine (23), bromcresol green (24), and hydroxylamine followed by ferric chloride (1).

Isolation and Identification of 2-Deoxy-D-gluconate

Preliminary experiments indicated that the oxidation of 2-deoxy-D-glucose could be carried out in the absence of any buffer. Therefore, for the preparation and isolation of the product from the oxidation of 2-deoxy-D-glucose no buffer was used. A total of 500 μmoles 2-deoxy-D-glucose in 10 Warburg flasks was oxidized in the presence of the particulate enzyme. After about 2 hours the consumption of oxygen reached the theoretical required for 0.5 mole per mole of substrate. The reaction mixtures were combined and diluted with 1 volume of water and applied to a coconut charcoal column. The column was prepared as follows. One hundred grams of charcoal was placed in a glass column with a sintered glass bottom and washed successively with 6 *N* HCl, water until neutral, ethanol, water, 200 ml 1% ammonia, and water until neutral. The sample was added to the column and the column washed with water containing 7% ethanol to remove inorganic salts and unchanged 2-deoxy-D-glucose. The 2-deoxy-D-gluconate was eluted with 1 liter of 17% ethanol, and fractions of 100 ml were collected. The last seven fractions, containing the 2-deoxy-D-gluconate, were combined and evaporated to dryness *in vacuo* at 45° C. The product obtained was compared with chemically prepared 2-deoxy-D-gluconate by paper chromatography and by infrared spectra of the barium salts.

Results and Discussion

Oxidation of Various Substrates

The preparations used in this work were more active on D-gluconate than D-glucose (Table I); the opposite result was obtained with lyophilized preparations (7). The results presented in Table I show 2-deoxy-D-glucose to be oxidized by the particulate preparation with a rate comparable to the oxidation of D-glucose. In view of the conclusion of Dalby and Blackwood (7) that the oxidation by the particulate fraction was specific for the D-glucose configuration at carbon-2, the utilization of 2-deoxy-D-glucose as a substrate was an unexpected result. The result suggests that the enzyme system was not specific for the D-glucose configuration, and that the low activity with compounds having configurations at carbon-2 different to D-glucose, such as mannose and glucosamine, may be due to steric factors. An alternative explanation for the result may be offered. Williams and Eagon (39) have found that cell-free extracts of *Pseudomonas aeruginosa* catalyze the oxidation of D-glucose to 2-keto-D-gluconate and also oxidize 2-deoxy-D-glucose to 2-deoxy-D-gluconate. In experiments on enzyme fractionation, they obtained a fraction active on 2-deoxy-D-glucose but not active on D-glucose. In the present studies we have observed that when the particulate preparation was stored for 2 months at

TABLE I

Oxidation of various substrates by a particulate preparation of *Aerobacter aerogenes*

Column 1	Relative rate	Column 2*	Relative rate
D-Glucose	100	D-Glucose	100
D-Gluconate	350	D-Gluconate	16
6-Deoxy-D-glucose	87	D-Allose	83
2-Deoxy-D-glucose	94	D-Mannose	13
D-Glucosamine	17	D-Galactose	13
D-Glucuronate	10	3-Methyl-D-glucose	18
D-Glucosaccharate	8	D-α-Fructoheptose	65
6-Deoxy-6-fluoroglucose	94	D-Xylose	20
Melibiose	17	D-Ribose	9
Gentiobiose	16		
DL-Lactate	255		
DL-Glycerate	26		
DL-Malate	15		

NOTE: The following were found to be inactive: L-arabinose, D-mannonate, L-rhamnose, D-altrose, maltose, cellobiose, lactose, DL-phenyllactate, glycollate, glyoxylate, and serine.
Conditions as described under Materials and Methods using phosphate buffer, pH 6.0.
*Values given in column 2 were taken from Dalby and Blackwood (7).

−10° C, the activity obtained with 2-deoxy-D-glucose as the substrate decreased to approximately one-third of that obtained with D-glucose as the substrate. This result suggests the presence of separate enzymes for the oxidation of D-glucose and 2-deoxy-D-glucose, and that the enzyme responsible for the oxidation of D-glucose is more stable than the enzyme responsible for the oxidation of 2-deoxy-D-glucose.

The effect of 2-deoxy-D-glucose on the rate of oxygen taken up by the particulate preparation in the presence of sufficient D-glucose to saturate the enzyme was examined. Under these conditions, in the presence of separate enzymes, increased reaction rates may be expected unless limited by certain factors such as substrate competition or lack of hydrogen acceptors. In our experiments we were unable to demonstrate increased oxidation rates. This result, however, should not be taken as evidence against the existence of separate enzymes. Attempts to obtain further evidence for the existence of separate enzymes from fractionation experiments were impossible because of the insolubility of the enzyme preparation. In view of the evidence presented by Williams and Eagon (39) for the existence of separate enzymes for the oxidation of D-glucose and 2-deoxy-D-glucose in *Pseudomonas aeruginosa*, it is postulated that in the present studies the oxidations of 2-deoxy-D-glucose and D-glucose are carried out by separate enzyme systems rather than a single enzyme system lacking specificity for the D-glucose configuration at carbon-2.

Some of the compounds altered in carbon-6 of D-glucose were readily oxidized by the particulate preparation. Thus, 6-deoxy-D-glucose and 6-deoxy-6-fluoro-D-glucose were oxidized with rates similar to D-glucose (Table I). However, other compounds, such as D-glucuronate and the 6-substituted disaccharides, were poorly oxidized. The low activity on the latter compounds may be attributed to steric factors.

The oxidation of D-gluconate by the particulate preparation has been shown to involve an enzyme system different from that for the oxidation of D-glucose

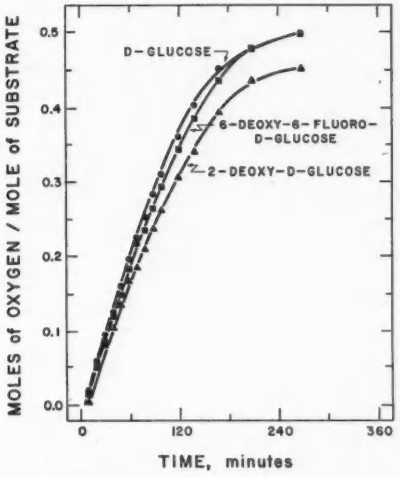

FIG. 1. Oxidation of 20 μmoles of D-glucose, 20 μmoles of 2-deoxy-D-glucose, and 20 μmoles 6-deoxy-6-fluoro-D-glucose by aged lyophilized preparations from *Aerobacter aerogenes*. Conditions are as described by Dalby and Blackwood (7).

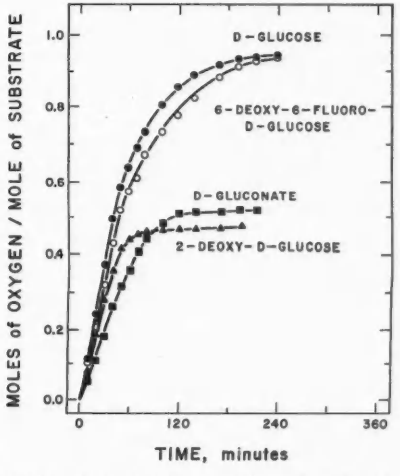

FIG. 2. Oxidation of 20 μmoles of D-glucose, 20 μmoles of D-gluconate, 20 μmoles of 2-deoxy-D-glucose, and 20 μmoles of 6-deoxy-6-fluoro-D-glucose by fresh lyophilized preparations of *Aerobacter aerogenes*. Conditions are as described by Dalby and Blackwood (7).

(7). In the present studies a particulate preparation, which was active on D-gluconate, oxidized D-glucose, 6-deoxy-D-glucose, or 6-deoxy-6-fluoro-D-glucose at similar rates and with an uptake of 1 mole of oxygen per mole of substrate (see Fig. 2). On the other hand, a lyophilized preparation, which had lost all activity on D-gluconate, oxidized D-glucose and 6-deoxy-6-fluoro-D-glucose with an uptake of only 0.5 moles of oxygen per mole of substrate (Fig. 1). D-Glucosaccharate was poorly oxidized, probably due to steric factors. Neither 2-deoxy-D-gluconate nor D-mannonate were oxidized, and 2-deoxy-D-gluconate was found to have no effect on the oxidation of D-gluconate. These results suggest that the gluconate-oxidizing system has a substrate specificity similar to the glucose-oxidizing system.

DL-Lactic acid was also rapidly oxidized by the preparations (Table I). DL-Glycerate was slowly oxidized, but DL-phenyllactate was inactive.

Oxidation of 2-Deoxy-D-glucose

As discussed above, 2-deoxy-D-glucose was not expected to be active as a substrate for the particulate preparation. The results in Fig. 1, obtained with a lyophilized preparation which had been stored several months, show that D-glucose and 2-deoxy-D-glucose were oxidized at comparable rates with an uptake of 0.5 moles of oxygen per mole of substrate. The preparation was inactive toward D-gluconate. The results in Fig. 2, obtained with a freshly lyophilized preparation, show that D-glucose was oxidized with an uptake of 1 mole oxygen while 2-deoxy-D-glucose and D-gluconate were oxidized with an uptake of 0.5 moles of oxygen per mole of substrate. These results suggest that the particulate preparation acts on 2-deoxy-D-glucose to form 2-deoxy-D-gluconate, which is not further attacked.

The product of the oxidation of 2-deoxy-D-glucose was isolated as described under Materials and Methods and compared with authentic 2-deoxy-D-gluconate. Examination of the isolated reaction product by paper chromatography showed the presence of two spots corresponding to 2-deoxy-D-gluconate

TABLE II

Chromatography of 2-deoxy-D-glucose oxidation product compared with 2-deoxy-D-gluconate

Compound	R_f value				
	Solvent A	Solvent B	Solvent C	Solvent D	Solvent E
2-Deoxy-D-glucose	0.61	0.46	0.33	0.68	0.35
2-Deoxy-D-gluconic acid	0.63	0.09	0.32	0.35	—
2-Deoxy-D-gluconolactone	0.63	0.57	0.32	0.35	0.27
Oxidation product of 2-deoxy-D-glucose	0.63	0.08	0.32	0.36	0.09
		0.55			0.27
Impurity in 2-deoxy-D-glucose			0.22		

SOLVENTS:
A—methanol:ethanol-water (45:45:10),
B—butanol:pyridine:water (50:20:18),
C—butanol:acetic acid:water (4:1:1),
D—ethanol:ammonia-water (8:1:1),
E—butanol:ethanol:water (4:1:5).

SPRAYS:
All compounds reacted with alkaline silver nitrate; 2-deoxy-D-glucose reacted with aniline phthalate; 2-deoxy-D-gluconic acid reacted with bromcresol green; 2-deoxy-D-gluconolactone reacted with hydroxylamine followed by ferric chloride.

and the lactone (Table II). The barium salt of the isolated reaction product and of the authentic 2-deoxy-D-gluconate were found to have identical infrared spectra with a peak at 1585 cm^{-1} which is ascribed to the salt of the carboxylic acid.

Since the demonstration by Woodward and his associates that 2-deoxy-D-glucose inhibits glucose fermentation by yeast (6, 40) and tumor glycolysis (41), several workers have used this inhibitor in metabolic studies with the assumption that 2-deoxy-D-glucose is inert except as an inhibitor. It has been shown that hexokinase converts 2-deoxy-D-glucose to 2-deoxy-6-phospho-D-glucose (40, 41), and the phosphorylated compound is reported to inhibit phosphoglucoisomerase (38). Extracts of *Leuconostoc mesenteroides* catalyze the reduction of TPN in the presence of 2-deoxy-6-phospho-D-glucose (10). Recently, 2-deoxy-D-glucose has been found to serve as a metabolic substrate in the growth of certain fungi (35). Because Williams and Eagon (39) have shown that extracts of *Pseudomonas aeruginosa* catalyze the conversion of 2-deoxy-D-glucose to 2-deoxy-D-gluconate, the present report is the second demonstration of a biological oxidation of 2-deoxy-D-glucose. In studies involving the use of 2-deoxy-D-glucose as a metabolic inhibitor, consideration should be given to the possibility of biological conversions.

Oxidation of 6-Deoxy-6-fluoro-D-glucose

Previous work (7) revealed that 6-deoxy-6-fluoro-D-glucose and D-glucose were oxidized by the particulate preparation with an uptake of 0.5 mole of oxygen per mole of substrate followed by a slower rate corresponding to the oxidation of D-gluconate. In the present work, both substrates were steadily oxidized with an uptake of 1 mole of oxygen per mole of substrate. The product was examined by paper chromatography and the results are shown in Table III. The product of the oxidation of D-glucose has an R_f value corresponding to 2-keto-D-gluconate and gives a yellowish green fluorescent spot with *o*-phenylenediamine, characteristic of 2-keto-D-gluconate (23). This confirms the suggestion made by Dalby and Blackwood (7) that 2-keto-D-

TABLE III

Chromatography of the oxidation products of glucose and 6-deoxy-6-fluoro-D-glucose

Compound	R_f	Reaction with: alkaline silver nitrate	Reaction with: *o*-phenylene-diamine
D-Glucose	0.46	+	Brown-black
D-Gluconate	0.18	+	—
2-Keto-D-gluconate	0.22	+	Yellow-green fluorescence
6-Deoxy-fluoro-D-glucose	0.62	+	Black-green
6-Deoxy-fluoro-D-gluconate	0.25	+	—
Oxidation product of glucose	0.24	+	Yellow-green fluorescence
Oxidation product of 6-deoxy-6-fluoro-glucose	0.35	+	Violet non-fluorescent

CONDITIONS: Twenty micromoles of substrate was incubated with the particulate preparation in the presence of 80 μmoles Tris–buffer, pH 6.0, and 30 μmoles $MgCl_2$. After the uptake of 1 mole oxygen per mole of substrate, the reaction mixture was treated with a few milligrams of trichloroacetic acid and 5 ml acetone. The precipitated protein was centrifuged off and the supernatant evaporated to a small volume. The product was examined by ascending paper chromatography with ethanol–ammonia–water (8:1:1) on No. 54 Whatman paper.

gluconate was the product of D-glucose oxidation. The product of the oxidation of 6-deoxy-6-fluoro-D-glucose was undoubtedly the 2-keto compound, although an authentic sample for direct comparison was unavailable. The oxidation product reacted readily with *o*-phenylenediamine, indicating that it was an α-keto acid, to give a non-fluorescent violet spot similar to that obtained for 2-keto-6-phosphogluconate (5, 8, 9, 27). We have assumed that the violet coloration is characteristic for 2-ketogluconic acids substituted at carbon-6.

6-Deoxy-D-glucose was oxidized with 1 mole of oxygen per mole of substrate similar to 6-deoxy-6-fluoro-D-glucose, to give a violet non-fluorescent spot with *o*-phenylenediamine after paper chromatography.

Oxidation of D-*Gluconate and* D- *and* L-*Lactate*

Preliminary studies showed that DL-lactate was oxidized nearly as rapidly as D-gluconate at pH 6.0, with the uptake of 0.5 mole oxygen per mole of substrate. Later studies showed that both D- and L-lactate were oxidized. The products of the reactions were identified as pyruvate by chromatography of the 2,4-dinitrophenyl hydrazones on paper. No pyruvate accumulated when the sonicate was used before ultracentrifugation, or when the supernatant after centrifugation was added to the particulate material. The conversion of pyruvate to acetolactate and acetoin in soluble extracts of *A. aerogenes* has been studied by other workers (14, 20).

From the results shown in Table IV, it can be concluded that the oxidation of D-lactate at pH 6.5 and L-lactate at pH 7.8 by the particulate fraction resulted in the uptake of 0.5 mole of oxygen and the production of 1 mole of pyruvate per mole of substrate. The lower than theoretical yield of pyruvate is undoubtedly due to contamination by enzymes catalyzing the conversion of pyruvate to acetolactate and acetoin.

Whereas the rates of oxidation of the D- and L-lactate by the cell extract before ultracentrifugation were approximately equal, the rate of oxidation of D-lactate by the particulate fraction was two to three times more rapid than L-lactate. This effect was found to be due to differences in pH, since the cell extract was strongly buffered at a higher pH.

A summary of the effect of pH on the oxidation of D-gluconate and D- and L-lactate is shown in Fig. 3. Phosphate buffer generally gave sharper peaks of activity than other buffers which were tried. For the lower pH values a mix-

TABLE IV

Oxidation of lactate to pyruvate by a particulate preparation from *Aerobacter aerogenes*

Substrate	Pyruvate recovered		μmoles oxygen taken up
	μmoles	% of control	
20 μmoles D-lactate, pH 6.5	15.4	85	10.1
20 μmoles pyruvate, pH 6.5	12.0	67	0
20 μmoles pyruvate added to blank, pH 6.5 (control)	18.0	—	—
20 μmoles L-lactate, pH 7.8	17.2	93	10.3
20 μmoles pyruvate, pH 7.8	18.6	92	0
20 μmoles pyruvate added to blank, pH 7.8 (control)	18.7	—	—

CONDITIONS as described under Materials and Methods.

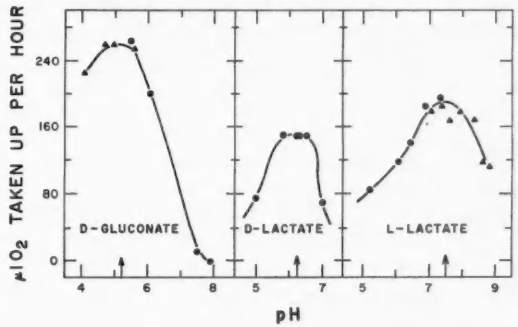

FIG. 3. Effect of pH on the oxidation of D-gluconate and D- and L-lactate by a particulate fraction from *Aerobacter aerogenes*.

ture of acetate–phosphate was used while for the higher pH values a mixture of Tris–phosphate was used. The values recorded are the final pH values. D-Gluconate was found to be oxidized most rapidly at approximately pH 5.2. D-Lactate was most rapidly oxidized in the range pH 6.0–6.5, but in several experiments a sharp maximum was obtained at pH 6.5. L-Lactate was most rapidly oxidized at around pH 7.8. Some difficulty was experienced measuring the activity of L-lactate at the higher pH values due to the high buffering action of the protein. Although the reason is unknown, the activity toward L-lactate relative to D-gluconate varied between different preparations.

When tested for lactic acid racemase, as described under Materials and Methods, no conversion of D-lactate to L-lactate could be detected. L-Lactate, incubated with the particulate material, was recovered unchanged. Therefore, the oxidation of these two substrates does not appear to be the result of a lactic acid racemase.

Oxidation of Mixed Substrates

The results presented above suggest that the particulate fraction contains three separate enzyme systems acting on D-gluconate and D- and L-lactate. A mixture of any two of the substrates therefore should result in a more rapid rate than either of the substrates alone. The results of experiments using two substrates show the rates approach the sum of the rates obtained for each substrate alone (Table V). Increasing the concentration of each substrate to equal the sum of two substrates resulted in only a slightly increased rate of reaction. Therefore, the increased rate observed in the presence of mixed substrates must be due to the action of separate enzymes acting on each substrate.

Effect of Inhibitors on the Oxidation of D-*Gluconate and* D- *and* L-*Lactate*

Dalby and Blackwood (7) demonstrated that the particulate fraction had a divalent metal requirement best satisfied by magnesium or calcium. In the experiments reported in this paper, 30 μmoles of magnesium was used. When magnesium was omitted from the reaction mixture, the activity with D-gluconate as substrate was markedly reduced (Table VI). The addition of

TABLE V

Oxidation of mixed substrates at pH 6.5 by a particulate preparation from *Aerobacter aerogenes*

Substrate	Rate of reaction (μl O_2/hour)	
	Found	Expected
30 μmoles D-gluconate	147	
60 μmoles D-gluconate	158	
30 μmoles D-lactate	160	
60 μmoles D-lactate	172	
30 μmoles L-lactate	138	
60 μmoles L-lactate	130	
30 μmoles D-gluconate + 30 μmoles D-lactate	290	307
30 μmoles D-gluconate + 30 μmoles L-lactate	286	285
30 μmoles D-lactate + 30 μmoles L-lactate	240	298

CONDITIONS as described under Materials and Methods.

TABLE VI

Effect of inhibitors on the oxidation of gluconate and D- and L-lactate by a particulate preparation from *Aerobacter aerogenes*

Inhibitor	Activity, % of control*		
	D-Gluconate	D-Lactate	L-Lactate
No magnesium added	48	92	100
Versene, 1.0×10^{-2} *M*	62	55	100
Washed in 0.05 *M* versene, pH 6.5; no magnesium added	32	55	98
Washed in 0.05 *M* versene; magnesium, 1.0×10^{-2} *M*	61	67	87
NaCN, 1.0×10^{-2} *M*	10	55	21
NaCN, 1.0×10^{-3} *M*	36	164	79
NaN_3, 1.0×10^{-2} *M*	46	175, 94	84
2,6-Dichlorophenolindophenol, 1.1×10^{-4} *M*	69	10	85
Riboflavin, 1×10^{-4} *M*	91	79	85
NaF, 1×10^{-2} *M*	104	130, 85	100
Arsenite, 1×10^{-2} *M*	100	100, 70	70, 104
Indoacetate, 1×10^{-2} *M*	102	105	114

*Control contained 1×10^{-2} *M* magnesium.

versene reduced the activity with both D-gluconate and D-lactate. The activity with L-lactate was not affected by these conditions. When the particulate preparation was washed with 0.05 *M* versene, pH 6.5, by ultracentrifugation in an attempt to remove metallic ions, the activity of the washed preparation on L-lactate in the absence of added magnesium was the same as the original preparation plus magnesium. The addition of magnesium to the washed preparation slightly lowered the activity on L-lactate. On the other hand, the activity of the washed preparation of D-gluconate and D-lactate in the absence of magnesium was markedly reduced and was increased in the presence of added magnesium, although the activity was not restored to the original value. The results in Table VI show that the oxidation of D-gluconate and D-lactate required magnesium for maximum activity while the oxidation of L-lactate is independent of magnesium.

The oxidation of D-gluconate was most sensitive to the action of sodium cyanide and sodium azide. While the oxidations of D-gluconate and L-lactate were always inhibited by these additions, the oxidation of D-lactate was frequently activated, particularly at lower concentrations of inhibitors. Sodium arsenite and sodium fluoride, while generally showing little effect, sometimes activated D-lactate oxidizing activity.

The activation of the oxidation of D-lactate by some of the inhibitors might be due to an inhibition of catalase. Catalase was present in large amounts in these preparations. In the absence of catalase, lactate is oxidized by 1 mole of oxygen resulting in the production of hydrogen peroxide and pyruvate, which react by the Holleman reaction (18) to form acetate, carbon dioxide, and water. In the presence of catalase, however, the peroxide is decomposed forming water and oxygen and resulting in the net uptake of 0.5 mole of oxygen per mole of substrate (37). In the present experiments, attempts to demonstrate the formation of hydrogen peroxide were unsuccessful. The addition of ethanol did not result in increased oxygen uptake (21), nor did the addition of *p*-aminobenzoic acid and horse-radish peroxidase result in the appearance of a color (12). The oxidation of D-lactate in the presence of 0.1 *M* sodium azide, which completely inhibited the action of catalase present in the particulate fraction, resulted in a rate 140% of the control containing no sodium azide. However, the total uptake of oxygen was the same in both cases. From the above observations it is difficult to come to any conclusions regarding the production of hydrogen peroxide. From similar observations, Madsen (25) has recently concluded that in the enzymatic oxidation of succinate, succinoxidase is "most likely a true oxidase". In the present studies it seems likely that the oxidation of these substrates is carried out by flavoprotein enzymes because they are inhibited by riboflavin (Table VI), which has been shown to be a potent inhibitor of the flavoprotein electron transport system (34). We found that 2,6-dichlorophenolindophenol was reduced by the particulate fraction in the presence of all three substrates. In Warburg experiments, 1.1×10^{-4} *M* concentration of the dye inhibited the rate of oxygen uptake (Table VI). Since the concentration of the dye is less than 2% of the substrate concentration no inhibition would be expected if the dye acted only as a hydrogen acceptor in place of oxygen. Therefore, it seems likely that the effect of the dye is to inhibit the transfer of electrons to oxygen. The larger inhibition in the presence of D-lactate further suggested a difference between the oxidation of D-lactate and the other two substrates.

There have been many reports in the literature on the isolation and study of specific D- and L-lactic acid oxidase (see reference 37) and dehydrogenases. Until recently there have been few reports of the occurrence of both activities in the same extract. Thus Nygaard, in a series of papers (see 28, 29, 30), and Boeri *et al.* (4) have described lactic acid dehydrogenases of yeast. Dennis and Kaplan (11) have isolated D- and L-lactic acid dehydrogenase from extracts of *Lactobacillus plantarum* (see also reference 22) and Molinari and Lara have described lactic acid dehydrogenase from *Propionibacterium pentosaceum* (26). Because of the insoluble nature of the enzymes in the present study, it is not possible to compare their properties with those described in the literature.

Acknowledgments

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INHIBITION OF A MICROBIAL GLUCOSE-6-PHOSPHATE DEHYDROGENASE BY DIETHYLSTILBESTROL[1]

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Abstract

Diethylstilbestrol readily inhibits the reduction of triphosphopyridine nucleotide by a glucose-6-phosphate dehydrogenase prepared from fractionated cell-free extracts of *Aerobacter aerogenes*. Hexestrol and dienestrol exert a similar influence but the dehydrogenase system was not sensitive to a number of steroids. The degree and extent of inhibition was a function of the diethylstilbestrol concentration. The inhibition could not be reversed by the addition of excess glucose-6-phosphate or triphosphopyridine nucleotide but the suppression could be relieved by the addition of increments of the fractionated extract containing the glucose-6-phosphate dehydrogenase enzyme. Several other enzymes such as isocitrate, alcohol, and malate dehydrogenases were found to be present in the same fraction but reduction of nucleotides by these enzymes was not influenced by the steroids or synthetic estrogen. A tentative hypothesis is that diethylstilbestrol may interact directly with certain enzymes, under specified experimental conditions, thereby regulating the rate of the enzymatic reaction. These findings thus suggest yet another mechanism whereby a synthetic estrogen may regulate intermediary metabolism.

Introduction

The mechanisms by which various hormones regulate cellular metabolism still present many questions. Hochster and Quastel (4) were among the first to delineate a mechanism of hormonal control when they demonstrated that diethylstilbestrol could act as a hydrogen carrier in an experimental system containing manganese dioxide as the terminal acceptor. Further studies by Hochster and Quastel (5) indicated that the glycerophosphate dehydrogenase of yeast was very sensitive to ketosteroids and that the sensitivity of enzymes from different sources varied greatly. Other workers have reported that some steroids will mediate the enzymatic transfer of hydrogen between the pyridine nucleotides in certain biological systems (10, 12). Shacter (7) suggested that stilbestrol influenced the enzyme systems in the cell membrane responsible for the "active transport" of glucose into the yeast cell. More recently, Yielding and Tomkins (13) reported that a number of steroid hormones inhibited the reduced diphosphopyridine nucleotide oxidase from either microbial or mammalian sources. Progesterone, estradiol, or diethylstilbestrol have been reported to be effective inhibitors of a glutamic acid dehydrogenase prepared from mitochondrial extracts of rat or beef liver but the glutamic dehydrogenase activity in a crude extract from *Salmonella typhimurium* was not sensitive to the synthetic estrogen (14). Durham and Perry (1) reported that diethylstilbestrol inhibited the oxidation of certain substrates by whole cell suspensions of *Aerobacter aerogenes*. These inhibitions could not be re-

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versed with yeast extract, casamino acids, brilliant cresyl blue, methylene blue, cytochrome *c*, diphosphopyridine nucleotide, or riboflavin-5-phosphate (2). To obtain additional information on the hormonal control of biochemical reactions, cell-free extracts were prepared from *A. aerogenes*, fractionated with ammonium sulphate, and the dehydrogenase activity for glucose-6-phosphate, isocitrate, alcohol, and malate measured in the presence and absence of various steroids and estrogenic compounds. Preliminary investigations indicated that glucose-6-phosphate dehydrogenase, a triphosphopyridine nucleotide-dependent enzyme, was repressed by diethylstilbestrol (3); however, the mode of inhibition was not definitely ascertained. The mechanism by which this synthetic estrogen may control cellular metabolism has now been partially delineated and will be presented in this paper.

Materials and Methods

The organism used throughout this investigation was a strain of *A. aerogenes* incapable of utilizing steroids. Cells used to prepare the cell-free extracts were grown on nutrient agar at 37° C for 18–20 hours, washed twice, and resuspended in 0.01 *M* Tris buffer of pH 7.0. The cell suspension, containing approximately 1.4 mg dry cell weight per ml, was disrupted in the French pressure cell under 20,000 p.s.i. at a delivery rate of one drop per second. The debris was removed by centrifugation in the cold (2–4° C) at $30,000 \times g$ for 60 minutes after which 8 volumes of the supernatant fraction were treated with 1 volume of a 1% protamine sulphate solution of pH 7.2 and the precipitate removed by centrifugation at $10,000 \times g$ for 30 minutes. The supernatant was fractionated in the cold by adding solid ammonium sulphate with constant stirring until a 50% saturated solution was obtained. The precipitate was removed by centrifugation and discarded and additional ammonium sulphate added until a 70% saturated solution was attained. The precipitate was recovered by centrifugation, dissolved in 20 ml of 0.01 *M* Tris buffer of pH 7.0, and dialyzed against frequent changes of distilled water for 24 hours prior to use. This fraction was used throughout the investigation. Protein was determined with a modified Folin reagent (9).

The influence of steroids on the dehydrogenase activity of the fractionated extract was measured by following the reduction of nucleotides at 340 mμ in the Beckman DU spectrophotometer at room temperature. The following components were added to the cuvettes: 0.1 ml fractionated extract (containing the indicated protein concentration); 20 μmoles substrate; 0.2 μmole triphosphopyridine nucleotide (TPN); 200 μmoles Tris buffer; 1 μmole $MnCl_2$; 0.1 ml of dioxane or 1:1 propylene glycol:water (containing desired steroid concentration); and water to a total volume of 3.0 ml. In studies using diphosphopyridine nucleotide (DPN), 2 μmoles was added to the cuvette. The enzymatic reaction was started by addition of DPN or TPN with an addermixer which permitted mixing immediately upon addition of the nucleotide.

Results

Influence of Steroids and Synthetic Estrogens on the Dehydrogenase Activity of the Fractionated Cell Extract

The enzymatic activity of the fractionated extract for glucose-6-phosphate,

alcohol, malate, and isocitrate dehydrogenases was measured in the presence and absence of various steroids and synthetic estrogens. Preliminary investigations indicated that both the glucose-6-phosphate and isocitrate dehydrogenase were TPN-dependent enzyme systems while alcohol and malate dehydrogenases reduced DPN. Figure 1 shows the results obtained when glucose-6-phosphate dehydrogenase activity was measured in the presence and absence of the test compounds. These results indicated that neither estrone nor androsterone influenced the reduction of TPN by the glucose-6-phosphate dehydrogenase in either of the concentrations used in this investigation (2×10^{-4} and 4×10^{-4} mmoles per cuvette). However, results did indicate that diethylstilbestrol (1 to 4×10^{-4} mmoles per cuvette) inhibited the rate of TPN reduction by this enzyme and the degree of inhibition was dependent on the inhibitor concentration. Earlier studies indicated that in addition to an inhibition in rate there might also be a decrease in the quantitative reduction of the nucleotide; however, more recent experiments indicate this is not the case. These findings suggest that the inhibitor interacts with a component of the reaction system thereby introducing a rate determining factor.

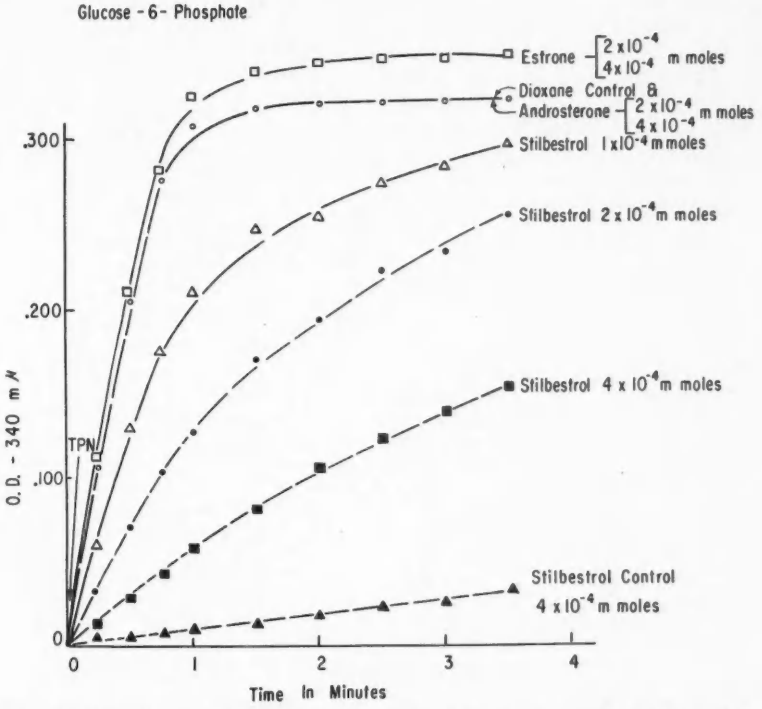

FIG. 1. Reduction of triphosphopyridine nucleotide (TPN) by glucose-6-phosphate dehydrogenase in the presence and absence of steroids and diethylstilbestrol.

Additional studies indicated that progesterone, testosterone, 17 α-estradiol, and 17 β-estradiol did not influence glucose-6-phosphate dehydrogenase activity. These compounds were used in concentrations of 2×10^{-4} and 4×10^{-4} mmoles per cuvette. When compounds structurally and functionally related to diethylstilbestrol, such as dienestrol and hexestrol, were used it was observed that they also inhibited TPN reduction by the glucose-6-phosphate dehydrogenase.

The influence of the test compounds on the reduction of TPN by isocitrate dehydrogenase was studied and the results are presented in Fig. 2. It was interesting to note that none of the steroids or synthetic estrogens tested with this enzyme system influence the rate of TPN reduction by isocitrate dehydrogenase even though this enzyme is also a TPN-dependent system. All compounds were tested in a final concentration of 4×10^{-4} mmoles per cuvette. The addition of DPN following completion of the reduction of TPN did not significantly change the optical density readings at 340 mμ. Thus the inhibition of nucleotide reduction apparently is a specific mechanism and is not a phenomenon characteristic of all TPN-reducing enzyme systems.

A series of control experiments were conducted in which the different components of the enzymatic system were added to the cuvette and the reaction followed by measuring the change in density at 340 mμ. Results indicated that the fraction containing the enzymatic activity was not capable of reducing TPN or DPN using the steroids as substrates and there was no significant change in density at this wave length when all components except TPN were mixed in the cuvette.

Spectrophotometric studies also indicated that the DPN-dependent ethanol

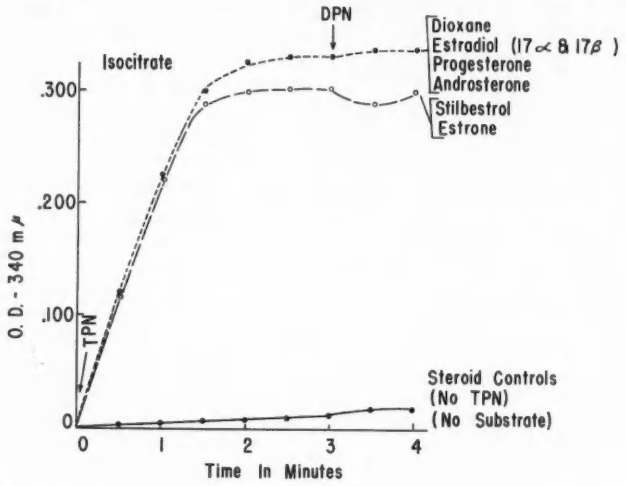

FIG. 2. Reduction of triphosphopyridine nucleotide (TPN) by isocitrate dehydrogenase in the presence and absence of steroids and diethylstilbestrol.

and malate dehydrogenases were not inhibited by the steroids and synthetic estrogens. Both of these enzymes were present in the fractionated extract as were the glucose-6-phosphate and isocitrate dehydrogenases. The results indicate that of the four dehydrogenase systems studied in this fraction of the extract, only the TPN-dependent glucose-6-phosphate dehydrogenase was sensitive to the synthetic estrogens and the inhibition was not observed in other TPN-dependent systems such as isocitrate dehydrogenase or in DPN-reducing systems such as malate and ethanol dehydrogenases. Similar findings were obtained with a glucose-6-phosphate dehydrogenase isolated from *Pseudomonas fluorescens*. Additional studies indicated that diethylstilbestrol did not influence the oxidation of DPNH.

Effect of Diethylstilbestrol when Added to Actively Reducing Systems

Studies were conducted to determine if diethylstilbestrol influenced the enzymatic activity when added to an enzyme system actively reducing TPN. All components except diethylstilbestrol were added to the cuvette and the reduction reaction initiated by the addition of TPN at 0 minutes. After 1½ minutes diethylstilbestrol was added to one system and dioxane was added to a second system to serve as a solvent control. The results from this study are presented in Fig. 3 and indicate that immediately following addition of the synthetic estrogen to the glucose-6-phosphate dehydrogenase system a decrease in the rate of nucleotide reduction is evident. In contrast to this observation is the finding that diethylstilbestrol did not influence TPN reduction by isocitrate dehydrogenase. These data augment the previous findings in that diethylstilbestrol does not influence all TPN-reducing systems in a similar manner and also suggest that the estrogen influences the enzymatic activity demonstrated by the fraction immediately following addition of the inhibitor to the reaction cuvette.

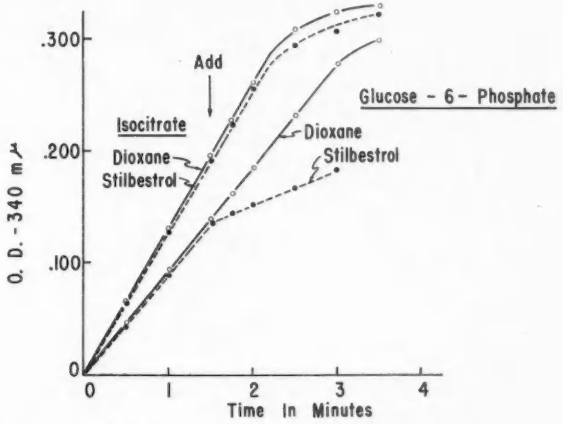

FIG. 3. Addition of diethylstilbestrol to active glucose-6-phosphate and isocitrate dehydrogenase systems.

Effect of Isocitrate Addition to a Diethylstilbestrol Inhibited Glucose-6-phosphate Dehydrogenase System

Additional studies were conducted to clarify the difference in sensitivity of the isocitrate and glucose-6-phosphate dehydrogenase systems to diethylstilbestrol. Experiments were designed in which isocitrate was added at a designated time interval to an active glucose-6-phosphate dehydrogenase system inhibited by different concentrations of diethylstilbestrol. Results from this study are presented in Fig. 4. The addition of isocitrate resulted in the immediate reduction of the available nucleotide by isocitrate dehydrogenase in those reaction vessels in which the glucose-6-phosphate dehydrogenase had been inhibited by diethylstilbestrol. The addition of water to one inhibited system served as a control. These results indicate that TPN in the oxidized state is readily available as an electron acceptor for the isocitrate dehydrogenase even though reduction of the nucleotide by glucose-6-phosphate dehydrogenase has been inhibited. These findings suggest that TPN is probably not the inactivated component in the diethylstilbestrol inhibited

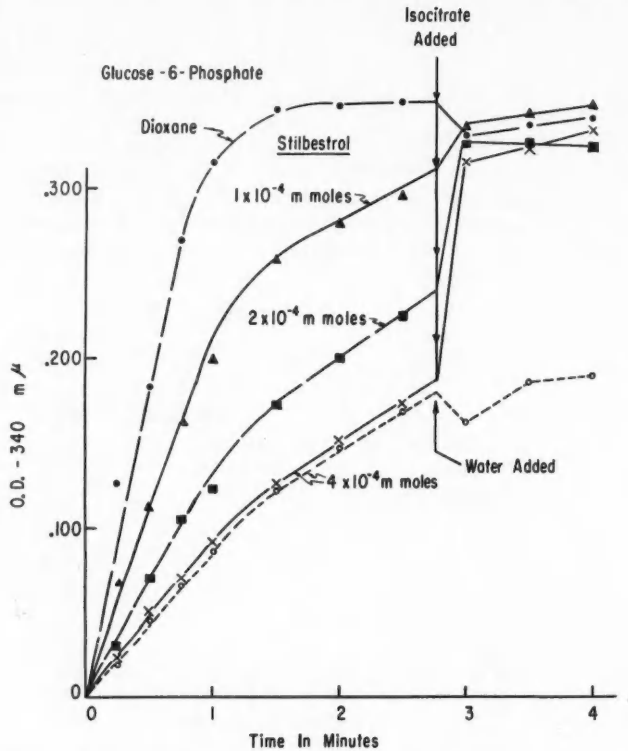

FIG. 4. Addition of isocitrate to a diethylstilbestrol-inhibited glucose-6-phosphate dehydrogenase system.

system but does not completely eliminate this possibility since isocitrate and glucose-6-phosphate dehydrogenase exhibit different stereospecificities and reduce the nucleotide by different mechanisms. It is possible that diethylstilbestrol could bind the nucleotide and prevent reduction by dehydrogenases in one plane but not influence reduction of the nucleotide by dehydrogenases that operate on the other side of the molecule.

Influence of Various Additives on TPN Reduction in an Inhibited System

Results obtained in earlier phases of this investigation indicated that diethylstilbestrol was inactivating one of the essential components of the reaction system. To test this possibility and identify the inactivated component, studies were conducted in which additional quantities of each ingredient of the enzymatic system were added to an active uninhibited TPN–reducing system (dioxane control) and a diethylstilbestrol-inhibited reaction system. Results obtained prior to and immediately following addition of the indicated components are presented in Fig. 5. These findings show that prior to addition of the indicated components the presence of diethylstilbestrol readily inhibited the reduction of TPN by glucose-6-phosphate dehydrogenase. The addition of TPN (0.1 μmole) or water (0.1 ml) did not significantly influence reduction of the nucleotide in either the control or diethylstilbestrol-inhibited system. However, addition of fractionated extract containing the enzyme (0.1 ml) apparently reversed the inhibition characteristic of the diethylstilbestrol containing system since the rate in the inhibited system appeared to approach the rate in the control cuvette. These findings would suggest that the enzyme may be the inactivated component in the inhibited system.

To delineate this phenomenon, studies were conducted in which additional quantities of each ingredient of the reaction system were added to an active control and diethylstilbestrol-inhibited reaction system. Results from this

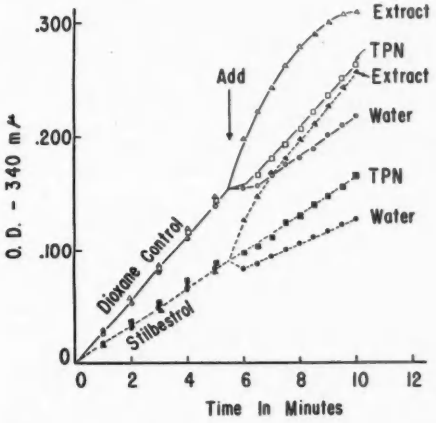

FIG. 5. Influence of various additives on nucleotide reduction in a control and diethylstilbestrol-inhibited system.

investigation are presented in Table I and indicate that diethylstilbestrol (1×10^{-7} moles) inhibited the basal rate approximately 47%. The addition of additional enzyme increased the reaction rate in the control ($\triangle$O.D. at 340 mμ from 55 to 94) and partially reversed the inhibition in the diethylstilbestrol system since the rate approached the control rate (from 29 to 85). The addition of diethylstilbestrol showed a pronounced inhibition in both systems as indicated by the drop in rate from 55 to 39 in the control system and from 29 to 16 in the system already containing diethylstilbestrol. The addition of TPN, substrate, or water did not significantly influence the rate in either system. Since the addition of these components did not influence the inhibition, these results suggest that the enzyme apparently is the inactivated component in the diethylstilbestrol-inhibited reaction system.

TABLE I

Influence of various additives on the rate of nucleotide reduction in a control and diethylstilbestrol inhibited system

	Propylene glycol control	Diethylstilbestrol (1×10^{-7} moles)
	Basal rate	
	55	29
	Rate after addition of indicated component(s)	
E	94	85
I	39	16
TPN	50	31
S	49	32
H_2O	51	28
E+I	53	34
E+TPN	89	81
E+S	96	84

NOTE: All components except the nucleotide were added to the cuvette and the reaction started by the addition of TPN. After the basal and inhibited rate were determined the indicated component(s) was added and reduction of the nucleotide followed until complete. The rates were calculated by measuring ΔO.D. at 340 mμ for a given time period (2 minutes). The rate was linear during this time interval. The following components were added to the cuvette: 0.1 ml fractionated extract (2.35 mg protein/ml); 20 μmoles glucose-6-phosphate; 0.2 μmole TPN; 200 μmoles Tris buffer, pH 7.8; 1.0 μmole $MnCl_2$; 0.1 ml of 1:1 propylene glycol:water (containing diethylstilbestrol in desired concentration); and water to a volume of 2.8 ml. All additives were used in the above concentrations in 0.1 ml quantities, the addition was made in a total volume of 0.3 ml and standardized by adding water and/or 1:1 propylene glycol:water. E = fractionated extract containing enzyme; I = diethylstilbestrol; TPN = oxidized triphosphopyridine nucleotide; S = glucose-6-phosphate.

Further studies were conducted in which mixtures of the various components were incubated together for 5 minutes prior to addition to the reaction vessel. Results from this phase of the investigation (Table I) showed that incubating the enzyme with either TPN or the substrate did not significantly increase the rate of TPN reduction over that obtained when the enzyme alone was added to the two different systems. However, when the fractionated extract containing the enzyme and the inhibitor were incubated together prior to addition to the reaction vessel the enzyme is no longer capable of producing the increased rate observed when the enzyme alone was added, although the rate is still greater than the reaction observed when only the inhibitor was added to the system. The decreased rate observed when the enzyme plus

inhibitor mixture was added to the reaction system indicates that the inhibitor is interacting with the enzyme, although the inhibition is not complete since a higher rate was observed in this system than the rate following addition of only the inhibitor. Protein determinations made following the incubation experiments did not show a significant difference when the extract was incubated in the presence and absence of the inhibitor. These findings suggest that the enzyme is the inactivated or limiting component in the diethylstilbestrol-inhibited system and the suppression can be relieved by incorporation of additional fractionated extract even though total protein determinations showed no significant difference in the presence and absence of the inhibitor.

Discussion

Studies have revealed that hormones and related compounds influence certain biochemical sequences at both cellular and subcellular levels. Results from this investigation indicate that diethylstilbestrol inhibits the reduction of TPN by glucose-6-phosphate dehydrogenase but does not influence the reduction of nucleotides by other dehydrogenases found to be active in the same fraction of the cell-free extract. The data also show that the addition of excess substrate or oxidized TPN did not reverse the inhibition. Since the excess TPN could not overcome the inhibition it would appear that diethylstilbestrol is not competing with this component in the electron transport system. These results were substantiated by the observation that addition of additional enzyme could reverse the inhibition. Related studies showed that incubation of the fractionated extract with diethylstilbestrol inactivated the enzyme, thereby decreasing the activity of the dehydrogenase. Thus, these findings suggest that in this experimental system diethylstilbestrol exerts its effect primarily on the glucose-6-phosphate dehydrogenase. Similar findings were observed by McKerns and Bell (6) using adrenal cortex preparations. These workers contended that estrogenic compounds inhibited the removal of hydrogen from glucose-6-phosphate by binding with the TPN-accepting sites on the dehydrogenase apoenzyme.

The specificity of the synthetic estrogen for the glucose-6-phosphate dehydrogenase is very interesting. One possible explanation might be the stereospecificity associated with reduction of the nucleotide by the enzymes since pyridine nucleotide dehydrogenases are known to be stereospecific (11). For example, glucose-6-phosphate dehydrogenase operates on the *beta* side of the nucleotide while isocitrate dehydrogenase operates in the other plane on the *alpha* side of the molecule (8, 11). Therefore, it would seem that diethylstilbestrol might inactivate those dehydrogenases that operate in the *beta* plane. This hypothesis could be substantiated by the report that diethylstilbestrol inhibits a reduced diphosphopyridine nucleotide – cytochrome *c* reductase which, like glucose-6-phosphate, involves the *beta* side of the molecule (13). Other enzymes operating on the *alpha* side include the alcohol, and malate dehydrogenases were shown to be resistant to the synthetic estrogen in this study. Unfortunately, attempts to demonstrate other dehydrogenase systems in this fraction that operate on the *beta* side of the nucleotide molecule have been unsuccessful, definite confirmation of the role of stereospecificity in the inhibition, if any, cannot be made at the present time. Another possible mech-

anism is that the estrogen reacts with specific active groups of a protein molecule thereby demonstrating a specificity for inhibiting certain enzyme systems. This hypothesis must also await additional study for confirmation.

Results from this investigation indicate that diethylstilbestrol affects glucose-6-phosphate dehydrogenase activity by interacting with the enzyme. The glucose-6-phosphate dehydrogenase of *P. fluorescens* was also sensitive; however, the specificity may vary in other biological systems. Hochster and Quastel (5) showed that α-glycerophosphate dehydrogenase obtained from a number of microbial and mammalian sources varied greatly in its sensitivity to various steroids. Yielding *et al.* (14) reported that a glutamic dehydrogenase isolated in crude extracts of *Salmonella typhimurium* was not sensitive to diethylstilbestrol but the glutamic dehydrogenase obtained from extracts of rat and beef liver was readily inhibited by the synthetic estrogen and other steroids. Thus the source of the enzyme and possibly the stereospecificity and active groups of the protein molecule may all play an important role in determining the sensitivity of proteins and enzymatic reactions to diethylstilbestrol and structurally and functionally related compounds.

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COMPARATIVE STUDIES WITH THE PHOTOOXIDASE OF RHODOSPIRILLUM[1]

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Abstract

When heterotrophically grown *Rhodospirillum rubrum* was reincubated anaerobically in the light, the photosynthetic growth resulted in a simultaneous appearance of the photosynthetic pigments, photooxidation, and photophosphorylation. Addition of exogenous diphosphopyridine nucleotide to extracts of photosynthetically grown cells inhibited photooxidation while photophosphorylation was stimulated. Ultraviolet irradiation inhibited photophosphorylation and added menadione relieved the inhibition. Photooxidase was less inhibited by this irradiation and menadione had no effect. No direct relationship between photooxidation and photophosphorylation could be shown.

Introduction

The metabolism of the light-generated reducing power in the chromatophore of *Rhodospirillum rubrum* is better documented than that of the light-generated oxidizing power. Reducing power has been shown to result in the reduction of diphosphopyridine nucleotide (6) and to participate in photosynthetic phosphorylation (5, 6). Though the demonstration of oxidizing power has been implied from existing data (4, 14, 15), the actual metabolism of the photooxidant is yet unknown. Two of the chromatophoral photochemical reactions, photooxidation and photophosphorylation, have been shown to disappear simultaneously when anaerobically light-grown *R. rubrum* was reincubated aerobically in the dark, or to appear simultaneously when aerobically dark-grown cells were reincubated anaerobically in the light (9, 13). This simultaneity suggested that these two reactions were either indirectly related through dependence on the photosynthetic pigments that are light and oxygen sensitive (2) or directly related with photooxidase, possibly functioning in energy liberation.

Materials and Methods

R. rubrum S1 was maintained as stabs in 1% yeast extract agar and was grown in the following liquid medium: 10 g Difco yeast extract, 10 g lactic acid (60%), 1 g K_2HPO_4, 0.2 g $MgSO_4$, 0.05 g $CaCl_2.2H_2O$, 0.05 g ferric ammonium citrate, 1 liter distilled water. The pH was adjusted to 8 with concentrated NH_4OH before sterilization. Photosynthetic cultures were grown at 25° C under anaerobic conditions illuminated with two 100-watt incandescent lamps for 3 days after a 2% inoculation of cells grown anaerobically in the light. Heterotrophic cultures were grown aerobically in the dark at 25° C for

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[2]National Science Foundation Cooperative Fellow.

5 days after a 2% inoculation of cells grown aerobically in the dark. All cell crops were checked for motility before being harvested by centrifugation, washed twice in 0.1 *M*, pH 7.0 phosphate buffer, weighed, and stored at −20° C for no longer than 1 month.

Extracts were prepared by hand grinding the cells with alumina and extracting with 0.1 *M*, pH 7.0 phosphate buffer. This brei was centrifuged at 14,000 × *G* for 10 minutes and then at 25,000 × *G* for 30 minutes to remove the debris. The chromatophores were harvested from the supernatant of the preceding centrifugations and washed twice in 0.1 *M*, pH 7.0 phosphate buffer by three centrifugations at 100,000 × *G* for 30 minutes. The pellet was finally resuspended in 0.1 *M*, pH 7.0 phosphate buffer to give a protein concentration of approximately 10 mg per ml.

The assay for photophosphorylation was carried out under nitrogen in Thunberg tubes with these additions: 20 μM NaF, 20 μM Na succinate, 20 μM $KHCO_3$, 20 μM $MgCl_2$, 10 μM adenosine diphosphate (ADP), 1 ml extract in 0.1 *M*, pH 7.0 phosphate buffer. After an incubation period of 15 minutes at 35° C in white light, the tubes were heated, and 0.1 ml of the reaction mixture was banded on Whatman No. 4 filter paper. The chromatogram was developed with a butyric–ammonium–water (66:33:1) solvent, and the bands located by ultraviolet light. Absorbing bands were cut out, eluted with dilute HCl, and the optical densities measured at 2590 Å (13). The concentration of adenosine monophosphate (AMP) permitted a correction for myokinase activity, and an ultraviolet clear area of paper was extracted for a blank.

The photooxidase assay of Vernon and Kamen (15) was used with reduced 2,6-dichlorophenol indophenol as the electron donor. Pigment concentration was measured after suitable dilution of clear extracts at 8600 Å for chlorophyll and 4900 Å for the carotenoid plus cytochrome. Protein was measured by the method of Gornall *et al.* (7). An aliquot of the undiluted extract was placed in a boiling water bath for 3 minutes to destroy the pigments, which would otherwise interfere with this assay. Addition of the undiluted biuret reagent dissolved the coagulated protein.

Nucleotides used were purchased from Pabst Laboratories, Phenol indophenol from General Biochemicals Incorporated, and Menadione from Merck, Sharp & Dohme.

Results

If photosynthetically grown cells of *R. rubrum* were reincubated aerobically in the dark, a progressive loss of pigments, photooxidation, and photophosphorylation occurred. If the procedure was reversed, a more regular and rapid gain occurred. Data in Table I show that all three characteristics measured returned simultaneously when heterotrophically grown cells were incubated photosynthetically. The negative value for photophosphorylation for the dark grown cells precludes a kinetic analysis of the rates of return. This negative value results from the correction necessary for the myokinase activity in these extracts (the AMP appearing is subtracted from the adenosine triphosphate (ATP) appearing). The optical density changes at 8600 Å represent changes in chlorophyll concentration, while the changes at 4900 Å represent primarily carotenoid concentration and secondarily a cytochrome

shoulder. In the light, strong aeration will suppress pigment concentration, photooxidase, and photophosphorylation. Cells grown in the light under strong aeration behaved in these assays as did cells grown aerobically in the dark.

Our next efforts were to determine if photooxidase were in some way related to photophosphorylation. Data in Fig. 1 represent the phenomenon invariably observed. Photooxidase is a rugged enzyme (15) so any physical or chemical agent applied to the extract usually inhibits photophosphorylation long before photooxidase is affected. The higher protein concentration in the crude extract exerts the usual protective effect. Photooxidase is not infinitely heat resistant since boiling destroys photooxidase activity, demonstrating that the reaction is probably not mediated by some free metallic ion.

TABLE I

Return of photooxidase, photophosphorylation, and pigments in extracts of dark-grown *R. rubrum*

Growth conditions	Photooxidase*	Photo-phosphorylation†	Pigment concentration‡	
			8600 Å	4900 Å
Dark grown, 96 hr	0.0	−2.1	0.36	0.30
Dark grown, 48 hr then:				
light grown, 24 hr	0.62	0.7	0.58	0.40
light grown, 36 hr	0.68	1.0	0.64	0.47
light grown, 48 hr	0.81	1.4	1.1	0.51
light grown, 72 hr	1.4	1.4	1.2	0.59

* μmole reduced 2,6-dichlorophenol indophenol oxidized/mg protein/hour.
† μmole adenosine triphosphate synthesized/mg protein/hour.
‡ Optical density/mg protein.

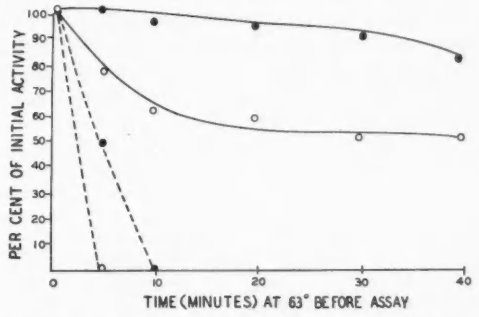

FIG. 1. Comparative heat sensitivity of photooxidase and photophosphorylation. ●———● Photooxidase of crude extract. ○———○ Photooxidase of chromatophore. ●- - -● Photophosphorylation of crude extract. ○- - -○ Photophosphorylation of chromatophore.

Two of the treatments applied did result in a differential effect on the two activites. Data in Table II indicate that exogenous oxidized diphosphopyridine nucleotide (DPN) depressed the photooxidase activity but stimulated photophosphorylation. This inhibition of the photooxidase assay could result from a photochemical reduction of DPN followed by a reduction, either

chemical or enzymatic, of the photooxidized indophenol. The stimulation of photophosphorylation is probably the result of a conservation of reducing power as discussed by Frenkel (5). Though the addition of DPN had a differential effect, it pointed out little except that this assay for photooxidase is distressingly sensitive and indirect.

TABLE II

Effect of added diphosphopyridine nucleotide on the activities of photooxidase and photophosphorylation

Conditions of assay	Photooxidation*	Photophosphorylation†
Plus 0.5 μM DPN	0.78	4.0
No added DPN	2.1	3.2

* μmole reduced 2,6-dichlorophenol indophenol oxidized/mg protein/hour.
† μmole adenosine triphosphate synthesized/mg protein/hour.

Of possibly greater significance was the effect of 3600 Å irradiation on these two reactions. Extracts were treated at minimum distance in open petri dishes on an ice bath with 3600 Å irradiation from a "Lite-Mite" lamp for 30 minutes. Controls were exposed in covered petri dishes. As shown in Table III photophosphorylation was strongly inhibited by the irradiation, and this inhibition was relieved by the addition of menadione. Photooxidation was only slightly inhibited and menadione had no effect. That the irradiation destroyed some of the photosynthetic pigments can be seen by the drop in the optical densities. This pigment destruction reduced the photooxidase activity but not the photophosphorylation activity. The assay for the latter reaction was designed to detect traces of photophosphory ation in the extracts from dark-grown cells. With a shorter assay period or a higher ADP concentration, a reduction in activity caused by pigment destruction should be apparent. Menadione has been suggested as an intermediate electron carrier in both oxidative and photosynthetic phosphorylation (1, 11). Menadione is apparently not involved in the metabolism of the photooxidant that reacts with the reduced indophenol.

TABLE III

Effect of 3600 Å irradiation and menadione on photooxidation and photophosphorylation

Treatment	Photooxidation*	Photophosphorylation†	Pigment concentration‡	
			8600 Å	4900 Å
No irradiation	0.92	0.76	2.2	1.1
Irradiated	0.67	0.28	1.7	0.79
Irradiated plus 0.5 μM menadione	0.67	0.77	1.7	0.79

* μmole reduced 2,6-dichlorophenol indophenol oxidized/mg protein/hour.
† μmole adenosine triphosphate synthesized/mg protein/hour.
‡Optical density/mg protein.

In a preliminary set of experiments, rates of activities of photooxidase and photophosphorylation were determined using red, blue, and white light. Though the final interpretation of these results depends on more accurate determinations of the light saturation curves than are now available, the ratio of the rates was essentially the same for all three light sources. This suggests that the same pigment system is necessary for both reactions.

Discussion

Of the two processes studied here, photophosphorylation is the better documented and understood. This esterification of phosphate apparently involves both the photoreductant and the photooxidant. The metabolism of the photoreductant can first be demonstrated by pyridine nucleotide reduction (6) and is further metabolized in photophosphorylation through an electron transfer system (5, 11). Photooxidation mechanisms are still a matter of conjecture as it is difficult to postulate mechanisms for the stabilization and transfer of "holes" rather than electrons. The carotenoids are presumed to be involved in the metabolism of the photooxidant (8), but no connection has yet been demonstrated between photooxidation and these pigments. Cytochromes are oxidized by photooxidation (3), but this doesn't indicate how the oxidizing power is first stabilized.

The hypothetical roles of photooxidase have ranged from insignificant to fundamental. The arguments for insignificance are based on the oxygen requirement for photooxidase in an anaerobe and on the heat resistance of the reaction. The oxygen requirement has been shown to be an artefact of extract preparation as originally stated by Vernon and Kamen (15). Sulphate (10), fumarate, and other oxidized molecules (16) have been shown to substitute for oxygen in the assay for photooxidase. The heat resistance of the chromatophoral photooxidase is significantly less than that of the crude extract. Even if this activity in the crude extract were resistant to boiling, photooxidation mediated by a free inorganic ion would still be of physiological significance and interest. Photooxidase has been postulated to function in oxidation of the external electron donor (2) or of a chromatophoral cytochrome (16) or to be directly related to energy production (see 12).

Our original hope was to show that photooxidase functioned in photophosphorylation much as does cytochrome oxidase in oxidative phosphorylation. We feel that the photooxidase is a chromatophoral cytochrome oxidase that uses the photooxidant as an electron depot. Unfortunately our evidence neither supports nor vitiates this hypothesis. A more direct assay for photooxidase will have to be worked out before a function can be assigned to this chromatophoral enzyme.

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SUBSTRATE-DEPENDENT PHOSPHORYLATION IN RESTING CELLS OF PSEUDOMONAS AERUGINOSA[1]

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Abstract

Resting cell suspensions of *Pseudomonas aeruginosa* exhibited substrate-dependent phosphorylation and most of the phosphate appeared in the nucleic acid fraction. The amount of P^{32} incorporated was a function of substrate concentration. When equivalent amounts of glucose, gluconate, or 2-ketogluconate were used as substrate, it was found that the more oxidized substrates supported appreciably less P^{32} incorporation, thus indicating that phosphorylation is coincident with the passage of electrons to oxygen by way of the electron transport chain. These data serve to illustrate that the practice of determining the amount of energy available from the dissimilation of a substrate by measuring growth yield can be in error since equivalent quantities of glucose, gluconate, and 2-ketogluconate supported equal amounts of growth. The P:O ratios obtained with glucose as substrate were of the order of 0.01. Phosphorylation was not sensitive to dinitrophenol or sodium fluoride but was completely inhibited by cyanide. Chloramphenicol, at a concentration which inhibited protein synthesis, caused a twofold stimulation of phosphate incorporation. Pyocyanine, which stops the oxidation of glucose at the 2-ketogluconate stage, completely inhibited phosphate uptake. The action of pyocyanine on both oxidation and phosphorylation could be reversed by magnesium. When extracts of this organism were studied, it was found that under all conditions the addition of oxidizable substrates decreased P^{32} incorporation.

Introduction

Our understanding of phosphate incorporation into the cells of aerobic microorganisms is still very incomplete and since very few species have been studied, no generalizations can be drawn. The oxidative activities of *Pseudomonas aeruginosa* have been found to be insensitive to dinitrophenol DNP (10) and the glycolytic pathway of glucose degradation is absent (2). It has been proposed that the pathway of glucose degradation involves gluconic and 2-ketogluconic acids (9). Since equimolar limiting quantities of these three compounds were found to give similar amounts of growth (3), it was concluded that the oxidation of glucose to gluconate or of gluconate to 2-ketogluconate did not result in concurrent phosphorylation. Since this organism has a full complement of cytochromes and the addition of glucose results in the reduction of cytochrome *c* (8), one could conclude that there is no phosphorylation during electron transport by way of the cytochromes even in growing cultures of *P. aeruginosa*. In view of the very limited information available on phosphate metabolism of *P. aeruginosa*, the present study was undertaken as a first step in understanding the mechanisms of phosphorylation in this organism.

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Materials and Methods

Pseudomonas aeruginosa A.T.C.C. 9027 was cultivated in the glucose–ammonium phosphate medium of Norris and Campbell (9) at 30° C for 20 hours in Roux flasks. Cells were harvested by centrifugation, washed once in cold distilled water, and suspended in 0.1 *M* tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.4, at a final concentration of 50 mg wet weight per ml.

Experiments with radioactive phosphorus were carried out in the Warburg respirometer. P^{32} as orthophosphate was tipped in from the side arm with substrate and carrier phosphate. The total reaction mixture (3.0 ml) contained approximately 0.1 μc of P^{32}. At the required time, duplicate 1-ml samples were removed from each flask and centrifuged and the supernatant discarded. The cells were then washed twice in cold distilled water, resuspended in ethanol, poured into planchets, dried, and counted.

For the preparation of cell free extracts (CFX), cells were washed once in distilled water and once in a 0.2 mg% solution of glutathione, frozen, and finally crushed in a Hughes press. The crushed cells were resuspended to a final concentration of 50 mg wet weight per ml in a Potter homogenizer at 0° C in a diluent composed of 0.05 *M* glycylglycine, 0.25 *M* sucrose, and 500 mg% egg albumin.

Phosphate incorporation studies with cell free extracts employed the following reaction mixture. Glycylglycine (50 μM/0.3 ml) plus orthophosphate (2 μM/0.3 ml) 0.5 ml; $MgCl_2.6H_2O$ (100 μM/ml) 0.5 ml; adenosine diphosphate (ADP) (40 μM/ml) 0.5 ml; cytochrome *c* (0.05 μM/ml) 0.2 ml; P^{32} (20 μc/ml) 0.2 ml; CFX 3.0 ml; substrate (100 μM/ml) 1.0 ml; sucrose (0.05 M) to 10 ml. In studies where a comparison was to be made between incorporation into whole cells and into CFX, it was found that the same reaction mixture could be employed, i.e. K_2HPO_4 (0.1 *M*) 0.15 ml; P^{32} (1.0 μc/ml) 0.1 ml; glucose (100 μM/ml) 0.05 ml; CFX or whole cell suspension, 1.0 ml; Tris buffer, pH 7.4 (0.1 *M*), to 3.0 ml. For determining the distribution of radioactivity among the various cell constituents, the fractionation procedure of Roberts *et al.* (11) was employed. Removal and estimation of the charcoal adsorbable material of the cold trichloroacetic acid soluble fraction were carried out by adding 20 mg of acid-washed Norit to 1 ml of the cold soluble fraction, washing the charcoal twice in cold water, and counting the washed charcoal. Further fractionation of the cold trichloroacetic acid soluble material with barium was accomplished according to the method of Umbreit *et al.* (12). Radioactivity measurements were made on a Nuclear Chicago scaler model 181A equipped with a gas flow counter. The production of adenosine triphosphate (ATP) was measured by the method of Kornberg (7).

Results and Discussion

Resting cell suspensions of *P. aeruginosa*, in which no change in bacterial numbers was detectable, nonetheless did exhibit substrate-dependent incorporation of P^{32} (Fig. 1). As an obligate aerobe, this organism has a relatively high rate of endogenous respiration resulting in the incorporation of appreciable quantities of P^{32}. However, uptake of P^{32} was increased almost threefold by the

presence of the oxidizable substrate, glucose. Most of the substrate-dependent incorporation can be accounted for by the increased P^{32} content of nucleic acid. This is in agreement with the results of Hotchkiss obtained with *Staphylococcus aureus* (5). In confirmation of earlier work on this organism (2) there was very little accumulation of sugar phosphate.

The amount of P^{32} incorporated into resting cells was a function of the amount of glucose oxidized (Fig. 2) and equivalent quantities of more oxidized substrates such as gluconate and 2-ketogluconate gave markedly lower P^{32} incorporation. At a substrate concentration of 2.5 μM per ml of reaction mixture, with glucose as substrate 0.053 μM of phosphate were taken up, whereas with gluconate the value was 0.044 μM and with 2-ketogluconate 0.031 μM. This information appears to be contradictory to the earlier work of Campbell *et al.* (3), in which it was concluded that equivalent quantities of these three substrates yielded similar quantities of available energy as evidenced by the fact that identical quantities of growth were obtained. The apparent discrepancy may be due to one of several factors. The absolute amount of phosphate involved in the experiments of Fig. 2 is of the order of 0.1 μM and so the differences may be too small to account for differences in amounts of growth. This does not seem likely for these small values reflect rather large differences in substrate concentration as shown by the fact that reaction mixtures containing 2.5 μM of substrate incorporate twice as much phosphate as those containing 1 μM. The other much more likely possibility is that there is a basic error in the assumption that equivalent amounts of growth are indicative of equivalent amounts of available energy. The carbon source performs two functions; one is as a source of energy and the other is as a

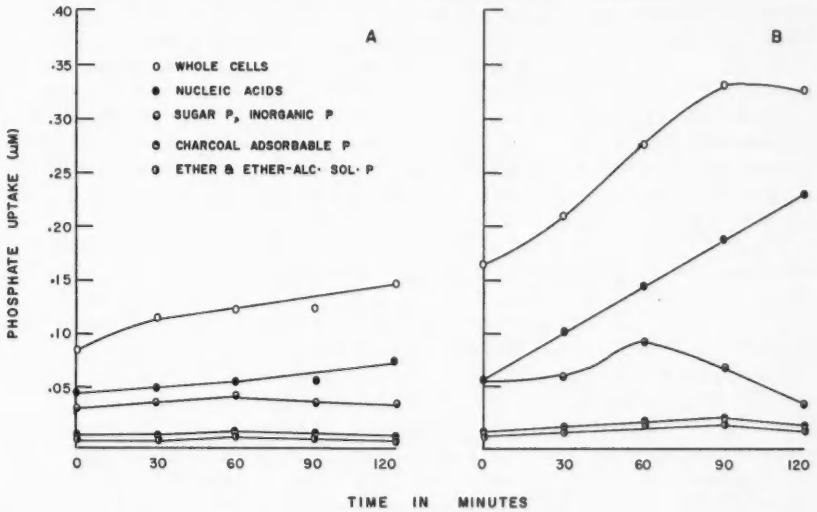

FIG. 1. P^{32} incorporation into resting cells of *P. aeruginosa*. (A) Endogenously respiring cells. (B) Cells with glucose as substrate.

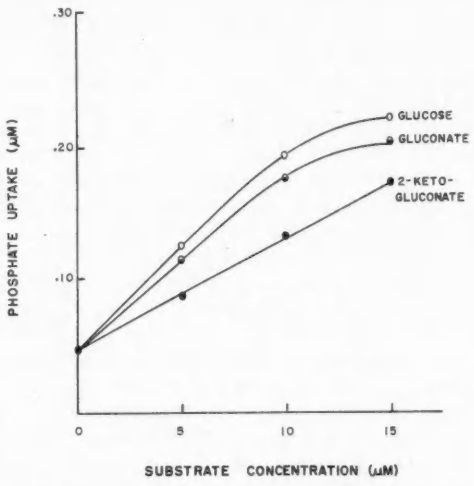

FIG. 2. P^{32} uptake as a function of concentration and degree of oxidation of substrate.

source of intermediates for anabolism. It could very well be that glucose, gluconate, and 2-ketogluconate yield different amounts of energy but that in no case is this limiting for growth. The limiting factor for growth might be the kinds and quantities of intermediates available for cell synthesis. If these three compounds are indeed part of the same metabolic pathway, then the kinds and amounts of intermediates formed from all three would be identical. Equivalent amounts of the three substrates would therefore support equal growth but would yield different quantities of energy and would incorporate different amounts of P^{32}. There is no evidence to suggest that the oxidation of glucose to gluconate or of gluconate to 2-ketogluconate generates energy at the substrate level so that differences in energy available from these compounds suggest that energy is gained by the passage of electrons to oxygen by the electron transport system of this organism.

The P:O ratios were of the order of 0.01 and were too low to be considered significant. These low ratios may be partly explained by the fact that, although there is no detectable change in bacterial numbers, there is incorporation of ammonia on the addition of oxidizable substrate (13) and presumably this reaction involves the expenditure of considerable amounts of energy.

As evidenced by the data of Table I, neither dinitrophenol (DNP) nor sodium fluoride appreciably changed the substrate-dependent incorporation of P^{32}. It does appear, however, that the endogenous incorporation of P^{32} was markedly decreased by fluoride and was slightly increased by DNP. By the use of the inulin space method, it was found that DNP freely penetrated the cell membrane of this organism (4). In contrast to these compounds, chloramphenicol, at a concentration which inhibited protein synthesis, caused almost a twofold increase in the rate of P^{32} incorporation. This observation is again in agreement

TABLE I

Effect of inhibitors on phosphate incorporation of resting cells of *P. aeruginosa*

Time (min)	Control		DNP (1×10^{-3})		NaF (1×10^{-2})		Chloramphenicol (20 μg/ml)		KCN (1×10^{-3})		Pyocyanine (1.5×10^{-3})	
	Phosphate uptake (μM/ml)											
	Endog.	Test	Endog.	Test	Endog.	Test	Endog.	Test	Endog.	Test	Endog.	Test
15	0.082	0.134	0.082	0.110	0.050	0.080	0.070	0.110	0.037	0.042	—	—
30	0.089	0.151	0.096	0.188	0.070	0.180	0.070	0.250	0.048	0.047	0.039	0.029
60	0.110	0.363	0.171	0.330	0.080	0.240	0.091	0.420	0 046	0.046	0.053	0.037
90	0.151	0.390	0.200	0.440	0.090	0.300	0.120	0.480	0.046	0.046	—	—

with the concept that protein synthesis causes a decrease in net phosphate uptake. The experiment with cyanide serves to emphasize that the incorporation of phosphate is not an exchange reaction but is dependent on the oxidative enzymes of the organism. Incubation of the reaction mixture in an ice bath gave similar data. In determining the effect of pyocyanine on phosphorylation, a concentration was used which was just sufficient to stop the reaction at the 2-ketogluconate stage (1) and under these conditions, no substrate-dependent incorporation of P^{32} occurred. The addition of magnesium reversed the inhibition of both oxygen uptake and P^{32} incorporation, thus suggesting that the ability of pyocyanine to uncouple oxidative phosphorylation is due to its ability to bind magnesium (6).

TABLE II

Influence of glucose concentration on the incorporation of phosphate by cell extracts

Glucose concentration (μM/ml)	Phosphate uptake (μM/ml)
0	0.024
0.004	0.017
0.040	0.017
0.400	0.017
4.000	0.017
40.000	0.017

The addition of an oxidizable substrate such as glucose to extracts of *Pseudomonas aeruginosa*, which had been prepared with either a Hughes press or a sonic oscillator, not only failed to stimulate phosphorylation but actually decreased it to a value well below that obtained with endogenously respiring extracts (Table II). Variations were made in the growth conditions, in the components of the reaction mixture, and in the method of rupturing the cells but in the more than one hundred individual experiments carried out, the addition of glucose always decreased net phosphate uptake. Gluconate, succinate, glutamate, and pyruvate, which were oxidized by extracts of this organism, also depressed phosphorylation. The use of the hexokinase trap (7) confirmed the observation that the oxidation of glucose by cell extracts did not result in the formation of ATP

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EFFECTS OF FREEZING, FREEZE-DRYING, AND STORAGE IN THE FREEZE-DRIED AND FROZEN STATE ON VIABILITY OF ESCHERICHIA COLI CELLS[1]

MARY T. CLEMENT

Abstract

Escherichia coli cells in concentrations of 2×10^9 cells per ml were resistant to freezing at −78° C and to low temperatures encountered during freeze-drying when suspended in distilled water, 7.5% glucose, 4.5% glycerol, skim milk, or serum but not when in saline. Survival immediately after freeze-drying varied with the drying interval and composition of the suspending medium and was highest (70–100%) in media containing 7.5% added glucose. Survival during storage in freeze-dried suspensions in serum containing 7.5% added glucose was inversely related to storage time and temperature; after 1 year at 32° C, 21° C, and 4° C, survival was 0, 25%, and 80% respectively. When suspensions in water, glucose, glycerol, and serum were frozen directly and stored in the frozen state at −18° C, −23° C, and −40° C, the cells showed a diminishing death rate and eventually attained a stable state. The final survival level varied with the composition of the suspending medium and the storage temperature. Glycerol provided most consistent protection (minimum survival 65%). Survival in water and glucose was inversely proportional to the storage temperature. Viability in water was higher than in glucose and was equal to glycerol in storage at −40° C (80% after 2 years).

Introduction

Suitable methods were required for the long-term preservation of bacteria in a stock culture collection. Published studies on the effects of freezing, freeze-drying and storage of microorganisms in freeze-dried and frozen suspensions frequently present conflicting evidence of damage. These differences are difficult to resolve because of the many variations in experimental factors and techniques. In the present work, resistance of *E. coli* cells to freezing, freeze-drying, and storage was re-examined under conditions applicable to the preservation of cultures. The experimental strain, methods of culture, and age and concentration of the cells were kept constant while the suspending fluids, temperatures, and time intervals were varied.

Materials and Methods

The experimental organism, *Escherichia coli*, NRC No. 482, was grown on blood-agar base (Difco) at 30° C. Growth from a 20- to 24-hour agar slant culture, emulsified in 5.0 ml of medium, provided a suspension containing 2 to 4×10^9 cells per ml. The various suspending fluids are listed in Table I. Unless otherwise stated in the text, procedures were as follows: aliquots of the suspensions (0.5 ml) were dispensed into sterile ampoules made of 7-mm Pyrex tubing having an 18-mm bulb blown at one end. The suspensions were frozen in air, glycol–water, or alcohol – dry ice baths at the required tempera-

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tures and thawed by being shaken in water at 45° C for about 10 seconds. For freeze-drying, the suspensions were shell-frozen in alcohol – dry ice at −78° C and then inserted individually, at suitable intervals, on a manifold maintained under pressure of 0.002 to 0.005 mm of mercury. The condenser fused to the vertical manifold was immersed in alcohol – dry ice. The suspensions were dried with the ampoules exposed to room temperatures for the intervals stated in the experimental section.

Viability was determined before and after all treatments. The contents of the ampoules were diluted quantitatively with distilled water and appropriate concentrations plated in triplicate in blood-agar base (Difco). Colonies from viable cells were counted after 3 days' incubation at 30° C. Survival after treatment is expressed as the percentage of the number of viable cells (colonies) from a similar aliquot of the original, untreated suspension. Analysis of results of a statistical experiment on the effects of freezing at −78° C in different media showed a standard deviation of ±8% in water, glucose, and serum and of ±12% in saline. In general, however, no significance was attached to less than 30% reduction in viability because of the many variables.

Experimental and Results

Resistance to freezing in relation to freeze-drying was determined in cell suspensions in various media, shell-frozen at −78° C, and thawed at 45° C. There was no appreciable loss in viability from this treatment except in saline solution (Table I). Viability in water was the same as in suspending media containing milk and serum colloids or glycerol. Similarly, viability was not

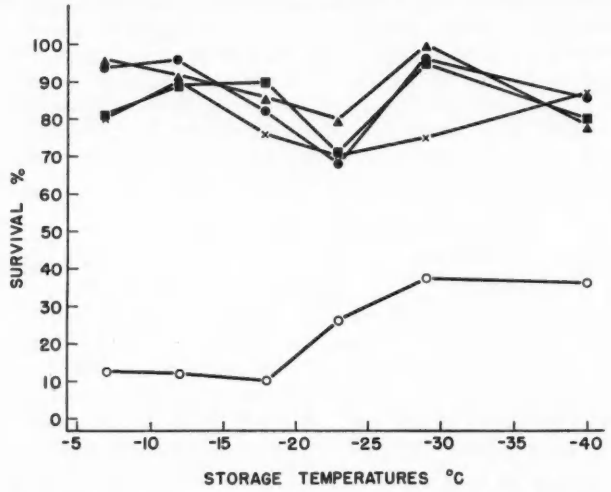

FIG. 1. Survival of *E. coli* cells in various media after freezing at −78° C and exposure to temperatures of −7°, −12°, −18°, −23°, −29°, −40° C for 24 hours. ▲ Distilled water; ● 7.5% glucose; ■ beef-blood serum; ○ 0.85% sodium chloride; × 0.85% sodium chloride + 7.5% glucose.

markedly reduced except in saline when suspensions frozen at −78° C were exposed for 24 hours to temperatures ranging from −40° C to −6° C (Fig. 1). Survival values in saline were reduced to about the same level (10%) at all temperatures above the eutectic point of sodium chloride, but the harmful effects were greatly diminished when 7.5% glucose was added to the saline.

Results of drying from the frozen state in the same suspending media (Table I) illustrates the lethal effects of drying in water and the excellent protection provided by glucose, either in aqueous solution or added to milk or serum. The extensive loss of viability shown in media without added glucose was mainly incurred during the first hour of drying. Viable counts on suspensions in serum and 7.5% glucose – serum removed from the manifold at intervals during freeze-drying showed that viability was well maintained in serum while the suspensions were in the frozen and partly dried state (Table II). Survival in this medium fell off sharply after most of the ice was removed and continued to decline after the preparations were visibly dry and at room temperature. In 7.5% glucose – serum, survival was maintained between 75 and 100% throughout the drying process.

Freeze-dried suspensions in 7.5% glucose – serum were stored at temperatures of 43° C, 32° C, 21° C, and 4° C, and viability determined at intervals up to 1 year (Table III). Results showed a direct relation between the death rate and storage temperature. After 1 year at 4° C, viability was the same as

TABLE I

Survival (%) of *E. coli* cells in various media after freezing and thawing and after freeze-drying for short and long intervals

Medium	After freezing at −78° C (2 min) and thawing at 45° C	After drying from the frozen state for intervals of: 1 hr	24 hr
Saline (0.85% NaCl)	43	0.4	0.01
Distilled water	95	2	0.7
Skim milk (12% Difco)	86	8	2
Beef-blood serum (Difco)	100+	29	15
Aqueous glucose (7.5%)	100	80	74
Aqueous glycerol (4.5%)	86	—	79*
Beef serum + 7.5% glucose	95	76	80
Skim milk + 7.5% glucose	100+	78	83

*Cells in glycerol residue (liquid) after removal of water.

TABLE II

Effect of drying time on survival (%) of *E. coli* cells in serum only and in serum containing 7.5% added glucose

Medium	After drying from the frozen state for: 10 minutes	20 minutes	35 minutes	45 minutes	180 minutes	1440 minutes
Beef serum only	80	89	59	55	49	27
Beef serum + 7.5% glucose	84	100	88	75	89	85

TABLE III

Effect of storage time and temperature on survival (%) of *E. coli* cells in freeze-dried suspensions in serum containing 7.5% added glucose

Storage temperature	After storage for:		
	2 months	6 months	12 months
4° C	78	74	76
21° C	75	55	25
32° C	0.2	0	—
43° C	0	—	—

immediately after drying, while at 32° C and 43° C the preparations were sterile after a few months and became dark brown and too insoluble for resuspension. Suspensions in serum, without added glucose, were more resistant at 32° C and 43° C (after 3 months average survival was 15% and 0.05% respectively) but were sterile after 1 year at these temperatures.

Resistance to storage in the frozen state was determined in suspensions in distilled water, 7.5% glucose, 4.5% glycerol, and 50% serum – water. Aliquots (0.5 ml) in 7×100 mm tubes were frozen at different rates in baths at −18°

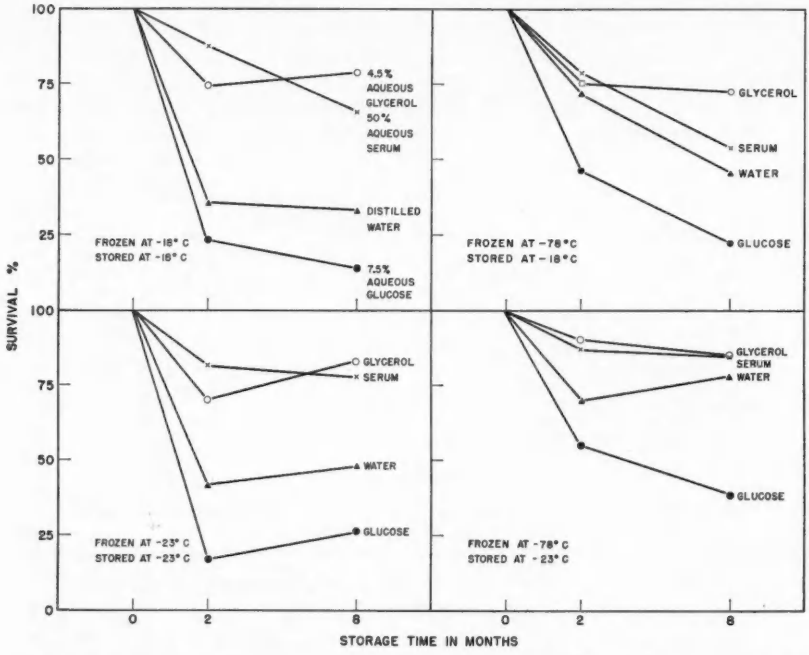

FIG. 2. Effects of freezing and storage temperatures and storage time on survival of *E. coli* cells in various media.

C, −23° C, −40° C, and −78° C, and then stored at −18° C, at −23° C (temperatures available in household freezers), and at −40° C. Survival was determined immediately (within 1 hour after freezing) and after storage for 2, for 8, and, in one treatment, for 28 months.

Viability was not appreciably affected by freezing and thawing (80 to 100% survival). After storage for 2 months in the frozen state, viability was well maintained in serum and glycerol at all temperatures and in all of the media in storage at −40° C (Fig. 2 and Table IV). An inverse relation was apparent between survival in water and glucose and the temperatures of freezing and storage. Many of the treatments showed no further decrease in viability after continued storage for 8 months. Viability declined during the latter interval mainly in suspensions in water, glucose, and serum, frozen at −78° C, and stored at −18° C (Fig. 2). While fast-freezing retarded storage death in water and glucose, survival in serum was lower in suspensions frozen at −40° C and −78° C (36% and 55%) than at −18° C (68%). Storage at −23° C after freezing at −78° C was equivalent to storage at −40° C. Glycerol provided most consistent protection for all freezing and storage temperatures.

Suspensions (in heat-sealed ampoules) frozen at −78° C showed no change in viable counts, except in glucose, after further storage for 2 years at −40° C (Table IV).

TABLE IV

Survival (%) of *E. coli* cells in frozen suspensions in various media stored at −40° C

Temperature of freezing before storage	Storage interval (months)	Suspending medium			
		Distilled water	Aqueous glucose 7.5%	Aqueous glycerol 4.5%	Aqueous serum 50%
−40° C	2	70	77	85	65
	8	69	46	81	64
−78° C	2	90	81	65	100
	8	78	70	70	72
	28	80	40	80	72

Discussion

Because of the unseparable effects of freezing and drying, it is impossible to obtain direct evidence of the extent to which prior freezing contributes to loss of viability during freeze-drying. There was no indication in the present work that appreciable damage resulted from exposure of the cells to freezing temperatures except in saline, which is not commonly used as a drying medium. High survival was shown in frozen and partly dried suspensions in serum but viability decreased with continued drying and further losses occurred after the preparations were visibly dry and at room temperature. Fry and Greaves (2) noted this direct relation between the drying time and loss of viability in albumin and other colloid media and recommended the addition of glucose, the optimal concentration being 7.5%. They attributed the beneficial effects of glucose to the retention of a critical amount of residual water that prevents

overdrying of the cells (1, 2). An association between viability and water removal would account for the wide variation in survival from suspending fluids differing in water-retaining properties and from techniques differing in water-removing capacity. Many of the factors affecting the rate and extent of water removal cannot be closely controlled and the presence of glucose reduces variability. The consistently high survival obtained after freeze-drying in 7.5% glucose – serum warranted further study of the effects of storage particularly at defined temperatures.

Unfortunately, rapid and progressive loss of viability occurred in this medium during storage at 43° and 32° C, followed by physical changes characteristic of browning. The formation and implication of carbonyl–protein complexes in dried cultures have been discussed by Scott (12, 13). It is claimed that damage to the cells can be inhibited or counteracted by the addition of carbonyl reagents or amino acids to the suspending medium, and that viability can be vastly improved by substituting a non-reducing sugar for glucose (12). While the results of Heckly *et al.* (5, 6) confirm the more rapid loss of viability in cultures lyophilized with glucose, the increase in storage life shown with sucrose is not of great practical significance; after 225 days, cultures of *Brucella melitensis* lyophilized with sucrose showed only 0.1% survival at room temperature and less than 10^{-4}% of the cells remained viable at 37° C. There was no loss of viability in sucrose at 0° C; however, similar stability was apparent in glucose–serum suspensions stored at 4° C in the present work. The relation between storage temperature and survival and the importance of cooling in maintaining viability have been illustrated by Scott with *Salmonella newport* dried in glucose buffer (13). Similar effects were reported by Proom with *E. coli* dried in papain digest (11) and by Maister *et al.* with *Serratia marcescens* in culture supernatant liquor (9).

In a recent publication Greaves cautions that because of the high moisture content, the addition of glucose is contraindicated for storage at high temperatures. When subjected to accelerated tests at 100° C, cells dried in media containing 5% sodium glutamate with either dextran, or albumin (3) showed superior resistance. While the retention of water undoubtedly contributed to the poor storage properties of glucose–serum, other factors must be responsible for the loss of viability in storage in the very dry preparations obtained in serum, and for the inverse relation between drying and storage life shown in similar colloid media, without added glucose (2).

Storage of bacteria in the frozen state was primarily recommended for the preservation of delicate species that do not withstand freeze-drying (7). However, broader application has been noted recently with the increasing availability of deep-freeze units and better understanding of the limitations of freeze-drying techniques. Glycerol has proved a suitable medium for low temperature storage of bacteria as well as for other types of cells (7, 8, 14, 15, 16). While glycerol provided best protection at harmful temperatures, in the present work survival in water was equivalent to glycerol in storage at −40° C and at −23° C after freezing at −78° C. Survival was consistently higher in water than in glucose and was greatly reduced in physiological saline. Harrison noted that survival of *E. coli* cells stored at −22° C was increased by decreasing the amount of (broth) solutes present and was best in distilled

water (4). Reports of death of bacteria at low temperatures frequently overlook the contributory effects of solutes, particularly metabolites and salts in isotonic and buffer solutions, which may become lethal to the cells after changes in concentration following ice separation and thawing. Conversely the value of glycerol and other protective adjuvants is most marked in saline suspensions because of their capacity to buffer salt damage to the cells.

There is much evidence that storage damage to living cells is decreased below −30° C (17) and temperatures of −40° C and −78° C are most commonly used for preservation. Microbiological assay organisms were successfully preserved in glycerol–buffer by direct freezing and storage at −40° C (16). Cells of *Treponema pallidum* were preserved in glycerol–saline without loss of virulence at −78° C but deteriorated rapidly at −40° C (7). Our results show that *E. coli* cells stored at −40° C were stable in water but not in glucose. At −78° C these effects were reversed and glucose became the better storage medium (unpublished data). Slow freezing and storage at −78° C are generally recommended for successful preservation in glycerol (4). Other factors contributing to stability are a constant storage temperature and a single freeze-thawing cycle. Fluctuations in temperature and repeated freezing and thawing are generally considered harmful although the degree of damage involved depends upon the composition of the suspending medium (7).

The diminished death rate and stable state attained during prolonged storage in frozen suspensions appear similar to effects observed by Major *et al.* (10) in broth suspensions of *E. coli* cells stored at −22° C. These authors reported that following a rapid initial destruction of the cells, the death rate diminished and after a time (2–3 weeks) a static state was reached.

The observation that freezing in itself is not very harmful and that storage death in frozen suspensions is not a function of time is favorable to the use of direct freezing as a preservation technique. On the other hand, there is increasing evidence that the more drastic and complex procedures employed in freeze-drying do not necessarily confer stability on the cells.

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NOTES

AN EXTRACELLULAR HAEMORRHAGIC TOXIN PRODUCED BY LISTERIA MONOCYTOGENES

PINGHUI V. LIU AND JANICE L. BATES

Although the infections with *Listeria monocytogenes* often result in the necrosis of the tissues and rapid death of the animals, the factor responsible for this process has not been produced in vitro. Since the bacterial cells of this species are relatively non-toxic, it is quite likely that some extracellular products of the organism were responsible for the necrosis and death of the animals. The infections by this species, therefore, were somewhat similar to the infections with *Bacillus anthracis* in which the animals usually die with signs of toxemia but the factor responsible for deaths of animals has not been demonstrated.

Recently, Smith, Keppie, and Stanley (1) demonstrated the production of an extracellular toxin of *B. anthracis* in vivo and the production of the same toxin in vitro was demonstrated by Harris-Smith, Smith, and Keppie (2). These workers found that the previous non-recognition of the toxin of *B. anthracis* in cultures was due to its early appearance and rapid disappearance under ordinary growth conditions. The possibility that the toxin production of *L. monocytogenes* may follow the same pattern has been investigated in this laboratory.

A strain of *L. monocytogenes* labelled KC 226 was obtained from the Communicable Disease Center, Chamblee, Georgia. The organism was grown on blood agar and then inoculated into a trypticase soy broth (Baltimore Biological Laboratories) so as to give about a barley visible turbidity. The broth cultures were incubated at 37° C for 6 hours and then centrifuged at $2000 \times g$ to eliminate the bacterial cells. The supernatant fluid was Seitz-filtered. When this fluid was inoculated intracutaneously into rabbits, no remarkable change was noted. However, when the fluid was concentrated 10 times and 0.1 ml was inoculated in the same way, a haemorrhagic lesion was produced within 3 hours, as shown in Fig. 1. The lesion became necrotic in 18 hours. When 0.5 ml of the preparation was injected intraperitoneally into mice, it killed the animals within 24 to 48 hours. Upon autopsy of these animals, focal necrosis of liver, similar to those produced by living cultures, was observed.

Two more strains of *L. monocytogenes* obtained from Dr. M. Hood (Department of Pathology, Charity Hospital of Louisiana) were also examined and the production of a similar toxin was demonstrated with both strains.

The haemorrhagic toxin was found to be heat labile, being inactivated by heating at 70° C for 30 minutes. It was not precipitated with 30% saturation of ammonium sulphate near neutral pH but could be precipitated by 3 volumes

of ethanol at −5° C. More work on the toxin is being conducted at present and the results will be reported later.

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THE SPECIFIC IDENTITY OF JENSENIA CANICRURIA

RUTH E. GORDON AND JOAN M. MIHM

The morphological similarity of *Jensenia canicruria* Bisset and Moore to *Nocardia rubra* (Krassilnikov) Waksman and Henrici was observed and reported by Adams and McClung (1). Gordon and Mihm (3, 5) assigned, to one species, 79 strains, among which were three received as *N. rubra* and two received as *Proactinomyces ruber* Krassilnikov. The oldest species represented among the 79 strains was *Micrococcus rhodochrous* Overbeck. The specific epithet *rhodochrous* was given to the species, therefore, according to the rule of priority (2). Because the morphology of these 79 strains, growing in the rough, smooth, or intermediate stages, was considered more like that of strains of mycobacteria than like the morphology of 98 strains of *N. asteroides* (*N. farcinica*), the type species of the genus *Nocardia* (3, 4), the species *rhodochrous* was tentatively assigned to the genus *Mycobacterium*.

Strain 8036 from the National Collection of Type Cultures, the type strain of *J. canicruria*, was examined recently and found to possess the morphology and the distinctive group of physiological characteristics of *M. rhodochrous*. On glycerol agar, its cells were pleomorphic rods or coccobacilli and did not retain the carbolfuchsin. Its undisturbed colonies on Bennett's agar, at 4 days, were dense with smooth edges or with a few outcroppings of short filaments. At 14 days, the filaments were fragmented into short forms. Aerial hyphae were not seen. The physiological characteristics of NCTC strain 8036 are listed in Table I and compared with those of the 79 strains previously assigned to the species *M. rhodochrous* (5). The report of Adams and McClung (1) is, therefore, confirmed. Strain 8036 is regarded as one of *M. rhodochrous* and *J. canicruria* as belonging in the synonymy of *M. rhodochrous*.

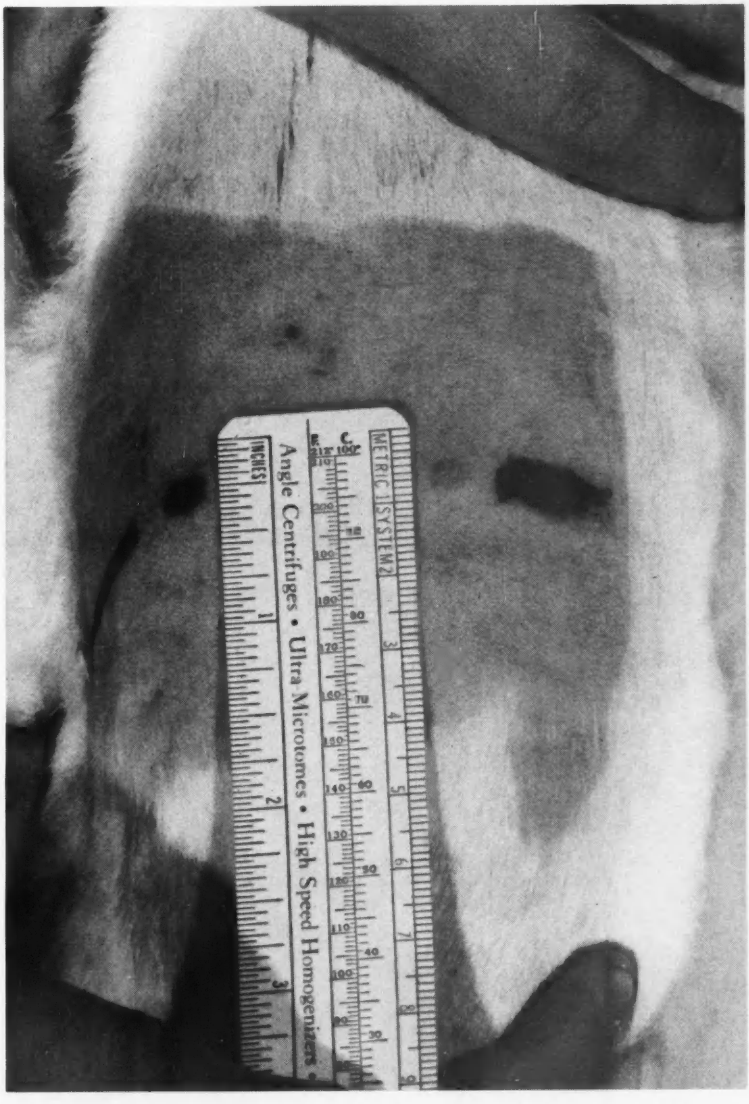

FIG. 1. The lesions produced in the skin of a rabbit by 0.1 ml of a 10 times concentrated supernatant fluid of a 6-hour-old broth culture of *Listeria monocytogenes* (KC 226). The lesion on the right side is 3 hours old. The lesion on left side is 18 hours old.

TABLE I

Comparison of physiological characteristics

Property	79 strains of *M. rhodochrous* (% positive strains)	Type strain of *J. canicruria*
Decomposition of:		
casein	0	−
tyrosine	76	+
Hydrolysis of starch	97	+
Growth at:		
52° C	3	−
45° C	54	−
40° C	76	−
35° C	90	+
28° C	100	+
10° C	100	+
Acid from:		
arabinose	0	−
dulcitol	0	−
erythritol	4	−
galactose	5	+
glucose	97	+
inositol	16	+
lactose	0	−
mannitol	100	+
mannose	100	+
α-methyl-D-glucoside	0	−
raffinose	0	−
rhamnose	8	−
sorbitol	100	+
trehalose	96	+
xylose	17	+
Utilization of:		
benzoate	80	−
citrate	87	+
lactate	99	+
malate	99	+
mucate	0	−
oxalate	0	−
succinate	100	+
Production of urease	75	+
Growth on the dyes:		
malachite green	1	−
methyl violet	0	−
pyronin	0	−
Color change of MacConkey agar	0	−

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